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Presence of *entA* and *entB* Genes among Thermophilic Bacteria Isolated from Hot Springs in Turkey

Gamze Başbülbül[1], Melihcan Ozteber[2], H. Halil Bıyık[2], Erman Oryaşın[1], Bülent Bozdoğan[1]

[1]*Recombinant DNA and Recombinant Protein Center, Adnan Menderes University, 09010, Aydin, Turkey*
[2]*Biology Department, Faculty of Arts and Science, Adnan Menderes University, 09010, Aydin, Turkey*

Abstract

Antibacterial activities of thermophilic bacteria were reported in previous studies. Whole genome sequences of thermophilic bacteria indicated presence of bacteriocin genes. In this study, 43 thermophilic bacteria isolated from hot spring, thermal mud and soil samples with antibacterial activity were screened for presence of 15 bacteriocin genes. Identification of thermophilic isolates was done by 16S rRNA gene sequencing. PCR reactions for bacteriocin gene screening were carried out by using primers specific for Pediocin, Plantaricin, Enterocin A, Enterocin B, Enterocin P, Enterocin Q, Enterocin L50 A/B, Enterocin AS-48, bact31, Cytolysin, Nisin, Lacticin 481, Lactococcin A, Enterolysin A and Lysostaphin genes. Sequence analysis indicated that thermophilic isolates were from the *Anoxybacillus* (n=18), *Geobacillus* (n=17), *Aeribacillus* (n=8) genera. The *entA* and *entB* structural genes were detected in 38 and 12 of 43 isolates, respectively. All isolates with *entB* gene also carried *entA* gene. Only 5 isolates were negative for both *entA* and *entB* genes. The results of sequencing and BLAST analyses of amplified genes showed homology with *entA* (91-100%) and *entB* (93-100%) genes. Our results showed that *entA* and *entB* bacteriocin genes are widely disseminated among these newly isolated thermophilic bacteria.

Keywords: Thermophilic, bacteria, bacteriocin, gene, PCR

Резюме

Антибактериалните активности на термофилни бактерии са публикувани и по-рано. Цялостните геномни последователности на термофилни бактерии показват наличието на бактериоцинови гени. В настоящето проучване са изследвани 43 термофилни бактерии с антибактериална активност, изолирани от горещи извори, термална кал и почвени проби за наличие на 15 бактериоцинови гени. Идентифицирането на термофилни изолати е извършено чрез 16S rRNA секвениране. PCR реакциите за скриниране на бактериоцинови ген са проведени с използване на праймери, специфични за педиоцин, плантарицин, ентероцин A, ентероцин B, ентероцин P, ентероцин Q, ентероцин L50 A/B, ентероцин AS-48, bact31, цитолизин, низин, лактицин 481, лактококцин A, ентеролизин A и лизостафин. Анализът на последователността показва, че термофилни изолати са от родовете *Anoxibacillus* (n = 18), *Geobacillus* (n = 17), *Aeribacillus* (n = 8). Структурните гени за entA и entB се откриват съответно в 38 и 12 от 43 изолати. Всички изолати с entB ген също така носят и entA ген. Само 5 изолати са отрицателни както за entA, така и за entB гените. Резултатите от секвенирането и BLAST анализите на амплифицираните гени показват хомоложност с entA (91-100%) и entB (93-100%) гени. Нашите резултати показат, че entA и entB бактериоциновите гени са широко разпространени сред тези новооткрити термофилни бактерии.

* Corresponding author:
Gamze Başbülbül, Adnan Menderes University,
Recombinant DNA and Recombinant Protein Center,
e-mail: gbasbulbul@adu.edu.tr

Introduction

Bacteriocins are ribosomally synthesized, small, heat-stable peptides produced by bacteria and with a narrow or broad spectrum (Cotter *et al*., 2005). The family includes a diversity of proteins in terms of size, microbial targets, modes of action and immunity mechanisms (James *et al*., 1991). According to Klaenhammer (1988), 99% of all bacteria may make at least one bacteriocin and the only reason more have not been isolated is that very few researchers have looked for them. Cotter *et al*. (2005) divided bacteriocins into two distinct categories: lanthibiotics (Class I) and non-lanthionine containing bacteriocins (Class II). Large, heat-labile murein hydrolases, which were formerly known as Class III bacteriocins, were termed ''Bacteriolysins''. Class IV bacteriocins were not included in this proposal because no members of this class have convincingly demonstrated yet. The most well-known examples of Lanthibiotics are Nisin and Lacticin 481, while pediocin, enterocins and lactococcin A are members of Class II bacteriocins.

In recent years, Franz *et al.* (2007), proposed a simplified classification scheme also for enterocins, bacteriocins produced by Enterococci; Class I enterocins (lantibiotic enterocins), Class II enterocins (small, non-lantibiotic peptides); Class III enterocins (cyclic enterocins); and Class IV enterocins (large proteins). Cytolysin belongs to Class I enterocins, and is a two-peptide bacteriocin with structural subunits contain lanthionie residues. Class II can be subdivided into three subclasses: II.1, enterocin of the pediocin family (enterocin A, enterocin P and bacteriocin 31); II.2, enterocins synthesized without a leader peptide (enterocin L50A/B, and enterocin Q); II.3, other linear, non-pediocin-type enterocins (enterocin B). Class III enterocins include cyclic antibacterial peptides like enterocin AS-48 and Class IV enterocins are large proteins such as enterolysin A.

Identification of a bacteriocin produced by a specific strain generally requires a complex process of biochemical characterization, purification and amino acid sequencing. Since the DNA sequences that encode the genetic determinants for the biosynthesis of some bacteriocins are known, they may be easily detected in various isolates by using PCR, instead of time and labour consuming methods (Rodriguez *et al*., 1997).

There is a growing interest in bacteriocins produced by LAB and other genera since these antibacterial substances have great potential in the food industry, veterinary or human medicine. Although reported bacteriocins are generally isolated from food-grade, mesophilic microorganisms, there are examples of bacteriocins produced by thermophilic bacteria and some members of archea (Novotny and Perry, 1992; Prangishvili *et al.,* 2000; Martirani *et al.,* 2002; Pokusaeva *et al*., 2009).

In this study, known bacteriocin structural genes which represent different classes of bacteriocins were investigated by PCR in thermophilic isolates from soil, thermal mud and water samples in Turkey. Our aim was to search the genetic determinants of antibacterial substances produced by thermophilic endospore forming bacteria.

Materials and Methods

Isolation and preselection of thermophilic bacteria

Samples of thermal pool mud, hot spring sediment and water and soil were collected aseptically from geothermal areas in Denizli, Mugla and Aydin provinces in Turkey and stored in sterile bottles or plastic bags. All samples were transported to the laboratory within 4 hours in a cooled container. At the time of sampling, the sites had temperatures varying between 23°C and 55°C, and a pH of 6.8 and 7.4.

Thermophilic bacteria were isolated by using *Thermus* medium at 65°C as described previously (Özdemir and Bıyık, 2012a; Özdemir and Bıyık, 2012b) and their ability to produce antibacterial substances against *Geobacillus stearothermophilus* DSM 22 was tested by a slighlty modified method.

Detection of antibacterial activity spectrum of selected isolates

To determine the antibacterial spectrum of fourty-three isolates, *Bacillus sphaericus* DSM 396, *B. thrungiensis* (soil isolate), *B. cereus* ATCC 11778, *Serratia marcescens* (soil isolate), *Escherichia coli* ATCC 35218, *Lactobacillus plantarum* DSM 20174, *Micrococcus luteus* ATCC 9341, *Staphylococcus aureus* ATCC 25923, *Bacillus* sp. (soil isolate), *Streptococcus vestibularis* DSM 5636, *B. mycoides* DSM 299, *Enterococcus faecalis* ATCC 51299, *Listeria monocytogenes* (food isolate) were used as indicator bacteria. Screening of antibacterial activities was done by the method mentioned previously (Özdemir and Bıyık, 2012).

16S rDNA sequencing and identification of bacteria

Genomic DNAs of thermophilic bacteria were isolated as described by Ronimus *et al*. (1997). 16S *rRNA* genes were amplified via PCR and the reaction mixture was as follows: 1X PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 1 U *Taq* polymerase

(all ABM, Canada), 0.4 µM of each of the following primers: 20F (5'- AGA GTT TGA TCC TGG CTC AG-3') and 1390R (5'- GAC GGG CGG TGT GTA CAA-3') (Orphan *et al.,* 2000) and water to give a final volume of 50 µl. One microlitre of extracted DNA was added to the mixture and the reactions were heated to 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 90 s. A final extension was carried out at 72°C for 15 min. Obtained amplicons were then sequenced by Macrogen (Korea) and the readings were manually edited by the use of BioEdit sequence editor software. Sequences were then compared with the reference strains of thermophilic bacteria held in Genbank. The sequence reads were between 990 and 1367 base pairs. The partial 16S *rRNA* gene sequences of isolates were submitted to GenBank Nucleotide Sequence Database and accession numbers as KC551235 to KC551272, KC167877, GQ255948, GQ487459 have been assigned.

PCR screening of bacteriocin genes

PCR detection of bacteriocin genes was performed using 1 µl of the DNA of each isolate, and amplification of genes was carried out using primers and conditions as mentioned in references (Cintas *et al.*, 2000; Garde *et al.*, 2001; Szwedaa *et al.,* 2001; DeVuyst *et al.,* 2003; Ghrairi *et al.,* 2007; Nigutova *et al.*, 2007; Yi *et al.*, 2009).

All the PCR reactions were done in a final volume of 50 µl containing 1x PCR buffer, 2 mM $MgCl_2$, 0,1 mM dNTPs, 1 U *Taq* polymerase (all ABM, Canada) and 0,5 µM of each of the forward and reverse primers. PCR products were resolved by electrophoresis on a 1.5% agarose gel. A negative control included in all PCR experiments and DNA positive controls were used to verify the presence of *entA, entB*, *entP*, *Bac31*, *L50 A/B* genes. Amplicons obtained by PCR were then sequenced by Macrogen (Korea) and the sequences were searched for a BLASTn homology in Genbank database.

Results

Totally 201 thermophilic bacteria were isolated in the study and fourty-three of them were able to inhibit the growth of *G. stearothermophilus* DSM 22. None of the thermophilic strain was found to be active against *E. coli, S. marcescens* or *S. aureus.* Nine of the fourty three isolates were active only against *G. stearothermophilus* DSM 22. *Listeria*- and *Enterococcus* active strains, which also inhibited the growth of other indicator bacteria, were also found among the thermophilic isolates.

According to 16S *rDNA* sequencing results, thermophilic isolates having antibacterial activity showed highest homology with bacteria belonging to genera *Anoxybacillus* (41.9 % of the isolates), *Geobacillus* (39.5%) *Aeribacillus* (18.6%) (Table 1). Analysis of the 43 partial sequences showed that one isolate (HBB 274) had similarity value of 97%, while the rest of the isolates scored between 98 and 100% to their most close relatives.

PCR screening of bacteriocin genes revealed the presence of e*nt*A and e*nt*B structural genes in 38 and 12 of the 43 isolates, respectively. As a result of BLASTn analysis, amplified e*nt*A genes showed sequence similarity values from 91 to 100% to other *entA* genes involved in the GenBank database. *entB* gene sequence similarities with those held in the database varied between 93 and 100%. All of the isolates that carried the *entB* gene were found to possess the *entA* gene as well. None of the Pediocin, Plantaricin, Enterocin P, Enterocin Q, Enterocin L50 A/B, Enterocin AS-48, bact31, Cytolysin, Nisin, Lacticin 481, Lactococcin A, Enterolysin A and Lysostaphin genes were detected in the thermophilic isolates.

Discussion

The 16S rRNA gene sequence analysis of the 43 thermophilic bacilli isolates conducted in this study revealed that the strains are members of the genera *Geobacillus, Anoxybacillus* and *Aeribacillus*. In 2001, thermophilic bacilli of phylogenetic group 5 were classified in a new genus, *Geobacillus,* by Nazina *et al*. (2001) with type strain *G. stearothermophilus*. Members of this genus are Gram-positive, spore-forming rods, neutrophilic, moderately thermophilic and aerobic or facultatively anaerobic and to date 19 *Geobacillus* species have been described (http://www.bacterio.cict.fr/g/geobacillus.html). The genus *Anoxybacillus* includes Gram-positive, alkaliphilic or alkalitolerant, thermophilic endospore forming bacteria (Miñana-Galbis *et al*., 2010. To date, genus *Anoxybacillus* has been represented by 19 species and two subspecies with validly published names (Goh *et al.,* 2013). In 2010, Miñana-Galbis *et al.* (2010) proposed that *G. pallidus* should be classified in a novel genus with proposed name of *Aeribacillus* according to results of 16S rRNA sequencing and phylogenetic analyses of genera *Geobacillus, Anoxybacillus* and strain DR03 isolated from hot spring in Mexico. *A. pallidus* is the only species within this genus that is also a type strain.

Thermophilic bacteria have become very im-

Table 1. Identification and source of thermophilic bacteria isolated in the study

Strain	Sample	Accesion no	% Identity	Taxon
HBB-10	Thermal sediment	KC551235	99%	*Aeribacillus pallidus strain MUST-1 [*JX535295]
HBB- 40	Thermal mud	KC167877	99%	*Geobacillus sp. O83* [DQ642093]
HBB-56	Thermal mud	KC551249	99%	*Anoxybacillus sp. DR04* [EU621362]
HBB-57	Thermal mud	KC551250	98%	*Anoxybacillus flavithermus* HBB-134 [GQ342689]
HBB-58	Thermal mud	KC551253	99%	*Aeribacillus pallidus strain MUST-1 [*JX535295]
HBB-59	Thermal mud	KC551236	99%	*Anoxybacillus flavithermus* HBB-134 [GQ342689]
HBB-60	Thermal mud	KC551237	99%	*Anoxybacillus flavithermus* HBB-134 [GQ342689]
HBB-105	Beach sand	KC551254	99%	*Aeribacillus pallidus strain MUST-1 [*JX535295]
HBB-106	Thermal mud	KC551255	99%	*Geobacillus* sp. Y4.1MC1 [CP002293]
HBB-110	Thermal mud	KC551271	99%	*Geobacillus* sp. Y4.1MC1 [CP002293]
HBB-143	Hot spring	KC551239	99%	*Anoxybacillus flavithermus* HBB-134 [GQ342689]
HBB-146	Hot spring	KC551238	99%	*Anoxybacillus flavithermus* HBB-134 [GQ342689]
HBB-148	Hot spring	KC551248	100%	*Anoxybacillus flavithermus* HBB-134 [GQ342689]
HBB-158	Hot spring	KC551251	99%	*Anoxybacillus flavithermus* HBB-134 [GQ342689]
HBB-160	Hot spring	KC551240	99%	*Anoxybacillus flavithermus* HBB-134 [GQ342689]
HBB-161	Hot spring	KC551273	99%	*Anoxybacillus flavithermus* HBB-134 [GQ342689]
HBB-180	Thermal mud	KC551241	99%	*Anoxybacillus flavithermus* HBB-134 [GQ342689]
HBB-182	Thermal mud	KC551242	99%	*Anoxybacillus flavithermus* HBB-134 [GQ342689]
HBB-183	Thermal mud	KC551243	99%	*Anoxybacillus flavithermus* AE3 [FN666242]
HBB-184	Thermal mud	KC551244	98%	*Anoxybacillus flavithermus* AE3 [FN666242]
HBB-211	Thermal water	KC551256	98%	*Anoxybacillus pushchinoensis AT-1 [*AB234214]
HBB-214	Soil	KC551257	99%	*Geobacillus sp. NBM49[*HQ703944]
HBB-215	Soil	KC551258	98%	*Geobacillus sp. NBM49[*HQ703944]
HBB-218	Soil	GQ255948	99%	*Geobacillus toebii* R-32652 [FN538991]
HBB-220	Soil	KC551259	99%	*Geobacillus sp. NBM49[*HQ703944]
HBB-225	Hot spring	KC551252	98%	Uncultured *Geobacillus* sp. clone ASC135 [JF825503]
HBB-226	Hot spring	KC551245	99%	*Anoxybacillus flavithermus* HBB-134 [GQ342689]
HBB-229	Hot spring	KC551246	98%	*Anoxybacillus sp. D1021 [*EU926955]
HBB-230	Hot spring	KC551247	99%	*Anoxybacillus flavithermus* HBB-134 [GQ342689]
HBB-234	Thermal mud	KC551260	99%	*Aeribacillus* sp. PZ-1[KC441060]
HBB-244	Thermal mud	KC551261	99%	*Aeribacillus pallidus* TSSA-3 [KC159167]
HBB-245	Thermal mud	KC551234	99%	*Aeribacillus pallidus strain MUST-1 [*JX535295]
HBB-246	Thermal mud	KC551262	99%	*Geobacillus* sp. Y4.1MC1 [CP002293]
HBB-247	Thermal mud	GQ487459	99%	*Geobacillus toebii* R-32652 [FN538991]
HBB-249	Thermal mud	KC551272	99%	*Geobacillus thermodenitrificans NG-80-2[*DQ243788]
HBB-269	Thermal mud	KC551263	99%	*Geobacillus* sp. Y4.1MC1 [CP002293]
HBB-270	Thermal mud	KC551264	99%	*Geobacillus* sp. Y4.1MC1 [CP002293]
HBB-271	Thermal mud	KC551265	98%	*Geobacillus sp. enrichment culture clone W2-5*[HM059722]
HBB-272	Thermal mud	KC551266	99%	*Geobacillus sp. enrichment culture clone W2-5*[HM059722]
HBB-274	Thermal mud	KC551267	97%	*Aeribacillus* sp. PZ-1[KC441060]
HBB-276	Thermal mud	KC551268	99%	*Geobacillus* sp. Y4.1MC1 [CP002293]
HBB-283	Thermal mud	KC551269	99%	*Geobacillus sp. NBM49[*HQ703944]
HBB-301	Soil	KC551270	99%	*Aeribacillus* sp. PZ-1[KC441060]

portant as the source of thermostable enzymes, and bacteriocins produced by these bacteria can also be useful in food biopreservation processes especially those requiring thermal treatment. Although there is a large amount of research on isolation and identification of thermophilic bacilli, studies related to bacteriocin production and its genetic determinants in thermophiles are very rare. Thermophilic bacteriocin producers reported in literature are *G. sterathermophilus* (Sharp *et al*., 1979), *Thermus ruber* (Becker *et al.*, 1986), *G. thermoleovorans* (Novotny and Perry, 1992), *B. licheniformis* (Martirani *et al*., 2002), *Sulfolobus islandicus* (Prangishvili *et al*., 2000); *G. toebii* (Özdemir and Bıyık, 2012a; Özdemir and Bıyık, 2012b).

The antibacterial activiy spectrum of strains isolated in this study was very diverse and targeted Gram-positive indicators, especially *Bacillus* spp. This situation is consistent with the fact that bacteriocins are more active to the bacteria closely related to the producer strain. When we investigated the genotypic basis of bacteriocin production, two bacteriocin structural genes, enterocin A and enterocin B were found in thermophilic isolates. Enterocin A, grouped as Class II pediocin-like bacteriocin, is produced by several *E. faecium* strains. Enterocin A structural gene is followed by an ORF and encodes the 103 aminoacid EntA immunity protein. EntA is active against *Enterococcus, Lactobacillus* and *Pediococcus* spp. and *Listeria* species including *L. monocytogenes* (Franz *et al.,* 2007). Our findins regarding the antibacterial activity spectrum of isolates were consistent with the data in the literature about enterocins. On the other hand, two strains, HBB-105 and HBB-246, which inhibited the growth of *L. monocytogenes* and *E. faecalis* in agar media, did not possess the *entA* or *entB* gene. New bacteriocins or other non-tested known bacteriocins could be responsible for the inhibitory activities in those isolates. Twelve thermophilic strains with *entB* gene carried *entA* gene also. Enterocin B is an additional bacteriocin produced by several Enterococci strains that produce enterocin A and it belongs to the non-pediocin type leaderless bacteriocin family of Class II enterocins. *entB* is possibly secreted by the dedicated transport proteins of EntA (which is the reason why the *entB* gene is always found associated with the *entA* gene), or some other transport system in the cell. *entB* is active against *Enterococcus* and *Lactobacillus* species and *L. monocytogenes*, *S. aureus* and *C. perfringens* (Casaus *et al.,* 1997).

Enterocins are very diverse bacteriocins and widely distributed among isolates from different sources. This situation can be explained by efficient gene transfer mechanisms of Enterococci and genetic determinants of many enterocins located on plasmids or conjugative transposons except for some examples like enterocin A and enterocin B. However, the same bacteriocin genes which are encoded chromosomally can also be found in different species, even in different genera. Shen *et al.* (2006) constructed individual genomic libraries from eight *S. pneumoniae* clinical isolates and as a result of sequencing studies they found that one of the isolate carried a mersacidin gene. Normally, mersacidin is known to be produced by *B. licheniformis* and its genetic determinant is located on the chromosome of the producer.

In recent years, lantibiotics and circular bacteriocins have been identified in the genomes of *G. thermodentirificans* NG80-2 and *G. stearothermophilus* DSM13240, respectively. A nisin analog encoded on the genome of the thermophilic bacterium *G. thermodentirificans* NG80-2 was obtained by heterologous expression in *E. coli* and the purified substance was termed geobacillin 1. The antimicrobial activity of geobacillin I was found to be very similar to Nisin A, while another antimicrobial compund, geobacillin II, produced in *E.coli*, only demonstrated antimicrobial activity against *Bacillus* strains (Garg *et al.,* 2012).

Kempermann *et al.* (2003) searched the genomes of *Staphlycococcus aureus*, *Oenococcus oeni* PSU-1 and *G. stearothermophilus* DSM 13240 for regions encoding novel, putative circular bacteriocins. These bacteria are not known to be circular bacteriocin producers, and researchers screened their genomes for orthologs of processing enzymes rather than of bacteriocins. Circularin A is a circular bacteriocin produced by *Clostridium beijerinckii* ATCC25752 and consists of five genes (cirABCDE). Circularicin A screening in protein and genome databases only resulted in a single hit on the chromosome of *G. stearothermophilus* DSM 13240, while three circularin C (which is the circularization protein in the production of circularin A) homologues were found to be encoded in the chromosomes of *S. aureus, O. oeni* and *G. stearothermophilus* DSM 13240.

It is clear that antimicrobial peptides produced by bacteria are more widespread in nature than it is currently known. These substances might be produced as a common ancestral property as a part of the defense systems in bacteria which represent different

taxonomic lineages. As a continuation of this study, amplification of sequences neighbouring the enterocin structual genes in thermophilic bacteria will provide detailed information about the arrangement of these genetic determinants.

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