African Journal of Biotechnology Vol. 10(3), pp. 439-441, 17 January, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1118
ISSN 1684–5315 © 2011 Academic Journals

Short Communication

Genomic study of the cereolysin A and B genes in *Bacillus cereus* isolated from raw and pasteurized milk

Samareh Fallah[1], Mohammad Chamani[2], Mehdi Aminafshar[2]* and Hamid Ezzatpanah[1]

[1]Department of Food Science and Technology, Faculty of Agriculture and Natural Resources, Science and research Branch, Islamic Azad University, Tehran, Iran.
[2]Department of Animal Science, Faculty of Agriculture and Natural Resources, Science and Research Branch, Islamic Azad University, Tehran, Iran.

Accepted 10 December, 2010

Bacillus cereus **spores are expanded in the nature and they can be separated from different foods. Cytotoxin is one of the most important poisons which is produced by *Bacillus cereus*, and it is highly resistant to heat and leads to diarrhea, nausea and vomiting syndrome. Hence study about existence of *Bacillus cereus* in pasteurized milk is very important due to probability of causing illness by Cereolysin gene products. Therefore, Different milk samples were collected from raw milk to pasteurized milk after various stages of producing pasteurized milk. Cultivation of milk samples in Mannitol egg yolk polymyxin B (MYP) media was done and it was followed by a purification of the observed colonies and investigation of cereolysin A and B by polymerase chain reaction (PCR) amplification. Thus, the gene was amplified and aligned with the previous sequences that were registered in the gene bank database. As such, many new missense mutations were observed at the amplified gene sequences. However these missense mutations caused a change in the sequence of amino acid at the protein chain but the protein efficiency and structure was not changed due to the substitution of amino acids with the same properties.**

Key words: milk- cereolysin - *Bacillus cereus*- polymerase chain reaction- gene.

INTRODUCTION

Bacillus cereus is a spore forming rod, catalase-positive, microaerophilic bacterium. Although it is a gram-positive bacterium, its gram property can be changed in a stationary phase of growth. On the other hand, *Bacillus* cultures are gram-positive when they are young, but may become gram-negative as they grow old (Anderson Borge et al., 2001; Shaheen et al, 2009). Aerobic sporulating *B. cereus* as a common microbial contaminant in various kinds of food may cause deterioration and spoilage in food product and food poisoning consumption, resulting in a socio-economical damage.

In fact, previous studies showed that *B. cereus* can affect the epithelial cells of the intestine and cause gastrointestinal diseases by producing entrotoxin (Gerit et al., 1998; Valik et al., 2003; Janštová et al., 2004). Many researchers have been conducted to determine *B. cereus* genes that produce entrotoxins, antibiotic and enzymes such as protease, amylase, phospholipase and hemolysin, so they can outbreak the disease by consumption of contaminated foods (Kotirantu et al., 2000; Hansen et al., 2001; Nduhiu et al., 2009). Dairy products including liquid milk (for example, pasteurized and UHT milk) may be contami-nated by *B. cereus* and as such, they probably contain some of its metabolites, especially *Bacillus* entrotoxins. Identification of *B. cereus* in raw milk and milk after diffe-rent process steps throughout the UHT processing plant was performed in order to investigate the capability of the applied processes in controlling *B. cereus* as a potential origin of entrotoxin.

One of the important genes of *B. cereus*, which is res-

*Corresponding author. E-mail: aminafshar@srbiau.ac.ir, aminafshar@gmail.com. Tel: +989121982407.

Abbreviations: Cer A, cereolysin A; **Cer B,** cereolysin B; **MYP,** Mannitol egg yolk polymyxin B; **PEG,** polyethylene glycol; **NCBI,** National Center for Biotechnology Information; **SNP,** single nucleotide polymorphism.

Table 1. Characteristics of the designed primers.

Gene	Direction	Primers	Annealing temperature (°C)
Cer A	Forward Reverse	5′ TTAACCGGACGACATCATAGG 3′ 5′ ACCTTTCACGTTTCATACCTCC 3′	56
Cer B	Forward Reverse	5′ GCTGAAGTTACACCAATGA 3′ 5′ CCACATTTTTCTCTCCTCT 3′	60

ponsible for disease, is cereolysin A and B (Cer A and Cer B). This gene encodes phospholipase C and sphingomyelinase, which constitute a biologically functional two component cytolysin. As such, *B. cereus* phospholipase C and sphingomyelinase act synergistically in lysing human erythrocytes (Schoen et al., 1999; Young et al., 2000; Granum et al., 1993; Perchat et al., 2005). According to the important role of the cereolysin A and B gene in food poisoning, amplification and characterization of them, in comparison with the recorded sequences in the database of the National Center for Biotechnology Information (NCBI) (accession number M24149.1), was considered as another purpose of this study. Also surveying the probable mutations and its effects on the characteristic of the cereolysin A and B protein was other aim of this study.

MATERIALS AND METHODS

Sampling and isolation of *B. cereus*

One hundred and eighty milk samples, including raw and pasteurized milk, were collected during two months. Samples were collected from raw milk and from the different stages of production line of pasteurized milk (raw milk receiving tank, reservoir balance tank, after cream separator and pasteurization). As such, raw milk was heated at the temperature of 80 °C for 10 min to eliminate some heat sensitive strain; however *B. cereus* was the heat resistant strain used in the study's samples (Svensson et al., 2000; Vyletelova et al., 2002). Samples were streaked on *B. cereus* selective agar base Mannitol egg yolk polymyxin B (MYP), and incubated under aerobic condition at the temperature of 30 °C (Vyletelova et al., 2002). Then purification of observed colony was done and the purified colony was cultivated in the Broth LB medium and incubated for about 48 hours.

DNA extraction

Genomic DNA was extracted from the purified *B. cereus* colonies that were cultivated in the broth LB medium. Total DNA was extracted from 2 ml of aliquot culture according to the modified Salting Out procedure (Miller et al., 1988). Extracted DNA was diluted in TE (Tris HCl 10 mM, Na2EDTA 0.2 mM, pH = 7.5) and the concentration was adjusted to 50 ng/µl. Electrophoresis on Agaros gel and Spectrophotometery were used to define the quality and quantity of extracted DNA.

Primer design and polymerase chain reactions (PCR)

Cereolysin A and B (CER A & CER B) are one of the important genes of B. cereous, which is responsible for food poisoning and disease. Therefore, in this research, two pair of primers were designed by Oligo software in order to amplify these genes by the polymerase chain reactions (PCR). Primers stringency and performance was checked, before PCR, using BLAST software at the NCBI webpage. As such, the Sequences of primers and their optimum annealing temperatures were showed in table 1.

Amplifications by PCR were performed in 30 µl volumes containing approximately 150 ng of DNA sample, 200 µM dNTPs, 1 U of Taq DNA polymerase, 1X PCR buffer, 1.5 to 3 mM MgCl2 and 0.5 µM of each primer. Consequently, PCR amplification was optimized for each primer separately. The PCR procedure was as follows: first denaturation step was done at 94°C for 3 min, 30 cycles of denaturation was done at 94°C for 30 seconds, annealing at primer-specific temperature for 30 seconds, extension at 72°C for 1 min and the final extension was done at 72°C for 10 min.

Electrophoresis and sequencing of the amplified gene

The electrophoresis of the PCR products was done on agarose gel, visualized on Ethidium Bromide, documented by Geldocumentation system, purified by polyethylene glycol (PEG) -precipitation and sequenced after purification using the cycle sequencing technology on ABI 3730XL 96-capillary sequencers. The sequence of Cer A and Cer B gene, related to the colonies of *B. cereus* in raw and pasteurized milk, was compared with the previous reported sequences at the National center for Biotechnology Information (NCBI) database (accession number M24149.1) and their mutations and variation were defined. Then the nucleotide sequence of the amplified Cer A and B gene was translated to the amino acid sequences in order to check the performance of these mutations. In the next step, the probable protein structure was made and aligned with the recorded protein structure at the NCBI database.

RESULTS AND DISCUSSION

The quality and quantity of the extracted DNA, related to the colonies of *B. cereus* in milk samples, were checked on an agarose gel. Both cereolysin A and B gene was amplified successfully. The length of Cer A and Cer B were equal to 858 and 1001 nucleotide, respectively. All detected mutations with their position on the cereolysin gene and their effect on protein substitution are shown in Table 2.

According to Table 2, nucleotide sequence of CerA showed fourteen single nucleotide polymorphism (mutation) in comparison with its sequence on the NCBI database (Accession Number: M24149) . Translation of the recent nucleotide sequence, into amino acid, showed only one probable amino acid change in the polypeptide chain. The observed single nucleotide polymorphism

Table 2. The observed single nucleotide polymorphism (SNP) of cereolysin A and B genes with their position on the gene (BN) and its effect on amino acid substitution (AAS).

Cer A	**SNP**	**C/G**	**G/C**	**T/G**	**A/G**	**A/T**	**T/C**	**G/A**	**G/A**	**T/C**	**A/T**	**A/G**	**G/A**	**T/A**	**T/C**
	BN	26	27	111	357	381	417	441	492	516	519	603	714	720	816
	AAS	-	Gly/Ala	-	-	-	-	-	-	-	-	-	-	-	-
Cer B	**SNP**	**T/C**	**G/A**	**A/T**	**A/T**	**C/T**	**G/C**	**T/C**	**T/C**	**C/T**	**A/G**	C/-	-/A		
	BN	86	87	190	225	255	276	333	763	824	930	937	976		
	AAS	-	Ala/Val	Phe/Ile	-	-	-	-	His/Tyr	Leu/Pro	-				

(SNP) on base number 27 may be replaced by glycine instead of alanine on cereolysin A amino acids chain. It can be concluded that, 13 mutations were silent in cereolysin A and did not have any significant effect on the amino acid chain sequences. In addition according to Table 2, ten SNP were detected in cereolysin B after sequencing and alignment with the registered sequence on NCBI database (Accession Number: M24149). However, six mutations were silent and did not show any expressions, but four SNP caused a substitution in valine instead of alanine, tyrosine instead of histidine, proline instead of leucine and isoleucine instead of phenylalanine respectively.

Many new missense mutations were observed at the amplified gene sequences. However, these missense mutations caused a change in the sequence of amino acid at the protein chain, but it was concluded that, the protein efficiency and structure was not changed due to the substitution of amino acids with the same properties.

According to the sequenced results of the cereolysin gene, it is concluded that, this strain potentially enters and possesses the genes that may be produced by the cereolysin protein, so that they can outbreak the disease by consumption of contaminated foods. However, it is suggested that the expression of cereolysin A and B genes on this special isolated strain should be surveyed in the future.

REFERENCES

Anderson Borge GI, Skeie M, Sørhaug T, Langsrud T, Granum PE (2001). Growth and toxin profiles of *Bacillus cereus* isolated from different food sources. Int. J. Food Microbiol. 69(3): 237-246.

Granum PE, Brynestad S, Kramer JM (1993). Analysis of enterotoxin production by *Bacillus cereus* from dairy products, food poisoning incidents and non-gastrointestinal infections. Int. J .Food Microbiol. 17(4): 269-279.

Hansen BM, Hendriksen NB (2001). Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. Appl. Environ. Microbiol. 67 (1): 185-189.

Janštová M, Dračková, L, Vorlová (2004). Influence of *Bacillus* spp. enzymes on ultra high temperature- treated milk proteins. Acta. Vet. 73: 393-400.

Kotiranta A, Lounatmaa K, Haapasalo M (2000). Epidemiology and pathogenesis of *Bacillus cereus* infections. Microb. Infect. 2(2): 189-198.

Miller SA, Dykes DD, Polesky HF (1988). A simple *salting out* procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16 (3): p. 1215.

Nduhiu J, Jackson NO, Dorcas WN, Michael MG (2009). Genetic characterization of food borne *Bacillus cereus* strains from milk, cheese and rice by multiplex PCR assay. IJIB. 5(2): 82-86.

Perchat S, Buisson C, Chaufaux J, Sanchis V, Lereclus D, Gohar M (2005). *Bacillus cereus* produces several nonproteinaceous insecticidal exotoxin. J. Invertebr. Pathol. 90(2): 131-133.

Schoeni JL, Wong AC (1999) . Heterogeneity observed in the components of hemolysin BL, an enterotoxin produced by *Bacillus cereus*. Int. J. Food Microbiol. 53(2-3): 159-167.

Shaheen R, Svensson B, Andersson MA, christiansson A (2009). Persistence strategies of *Bacillus cereus* spores isolated from dairy silo tank. Food Microbiol. Food Microbial. 27: 347-355.

Svensson B, Eneroth A, Brendehaug J, Molin G, Christiansson A (2000). Involvement of a pasteurizer in the contamination of milk by *Bacillus cereus* in a commercial dairy plant. J. Dairy Res. 67(3):455-460.

Valik L, Gorner F, Laukova D (2003). Growth dynamics of *Bacillus cereus* and shelf-life of pasteurized milk. Czech J. Food Sci. 21(6): 195-202.

Vyletelova M, Svec P, Pacova Z, Sedlacek I, Roubal P (2002). Occurrence of *Bacillus cereus* and Bacillus *licheniformis* strains in the course of UHT milk production. Czech J. Anim. Sci. 47(5): 200-205.

Young-Rok k, Czajka J, Batt CA (2000). Development of a fluorogenic probe-based PCR assay for detection of *Bacillus cereus* in nonfat dry milk. Appl. Environ. Microbiol. 66(4): 1453-1459.