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Non-Radioactive Labeled Probe Preparation for *hbs* Gene Detection

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Abstract: Among some *Bacillus* species, a protein highly homologous to HU, classified HB and coded by *hbs* gene. According to the recent studies, the sequence of *hbs* gene just in one strain of *Bacillus subtilis* exists in gene bank (ATCC 23857). In this study, DNA from *Bacillus subtilis* ATCC 6633 was extracted and investigated by PCR. The PCR product was sequenced and shown to differ in just one nucleotide from *B. subtilis* ATCC 23857. Hence, it was chosen as reference and for the first time, used for non-radioactive labeled probe preparation. The PCR product in *Bacillus subtilis* with ATCC 6633 was labeled using non-radioactive DIG-labeled nucleotides and conditions of probe preparation and hybridization were optimized and checked it by Southern blotting.

Key words: *Bacillus subtilis*, HBsu, *hbs* gene, DIG labeled DNA probe

INTRODUCTION

Histone-Like Proteins (HLPs) in bacteria are small, basic proteins that contribute to the control of gene expression, recombination, DNA replication and compression of the bacterial DNA in the nucleoid (Swinger and Rice, 2007). Among the HLPs, HU family proteins are widespread in prokaryotes and members of the family have at least 20% amino acid sequence identity (Dixon-Fly and Caro, 1999). Several proteins with properties and structures highly homologous to HU proteins have been isolated from other bacteria and bacteriophages. It seems that HU protein sequence on various strains of one species could be different. One of the best- studied histone like protein is HU of *Escherichia coli* (Ussery *et al*., 2001). The HU family has three classes, but the distribution is not uniform in Gram-negative and Gram-positive bacteria. Furthermore, whereas HU proteins from enterobacteria are predominantly heterotypic dimers, the HU proteins isolated to date from gram-positive bacteria are all homotypic dimers (Oberto *et al*., 1994; Yokohama *et al*., 1997). Among the HB proteins of different *Bacillus* species so far sequenced, conservation is more than 80% (Wolfgang and Marahiel, 2002).

Bacillus subtilis harbors only one histone-like protein, Hbsu, encoded by the essential *hbs* gene (Micka *et al*., 1991; Micka and Marahiel, 1992; Schmid, 1990). The HBsu protein shows homology to HU protein from *E. coli* and it has 57 and 52% identical amino acid residues with HU-2 and HU-1, respectively. Like HU, HBsu unspecifically binds to DNA. In contrast to HU, which forms predominantly heterodimers, HBsu binds DNA as a homodimer (Wolfgang and Marahiel, 2002).

The Hbsu is bound to SRP that is a long piece of RNA to which an additional protein (Yamane *et al*., 2004). Studies have determined the levels of HBsu in the spore, its localization in the forespore and explored the ability of HBsu to modulate the effects of a/b-type Small Acid-Soluble Proteins (SASP) on the DNase digestion, persistence length, supercoiling and UV photochemistry of DNA *in vitro* (Ross and Setlow, 2000). HBsu of the mesophilic *B. subtilis* and *Bacillus stearothermophilus* HU (BstHU) display 87% identity and 94% similarity together and so a nearly identical 3D-structure for both can be assumed (Wolfgang and Marahiel, 2002).

In this study, for the detection of *hbs* gene in some *Bacillus subtilis* strains, this gene was analyzed in *Bacillus subtilis* ATCC 6633 by PCR and this strain was chosen as reference. Whereas DIG-labeled probes maintain the detection sensitivity and furthermore, non-radioactive probe labeling and detection technology do not involve hazardous substances, the labeled probes are stable, can be stored for a long period and reused several times. For the first time, it was used for non-radioactive labeled probe preparation and the PCR product was labeled using of a non-radioactive DIG-labeled nucleotide following it which was used for the detection of *hbs* gene in this strain (ATCC 6633) by Southern Blotting.

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MATERIALS AND METHODS

This project has been conducted at Alzahra University since the middle of 2008.

Bacterial strain and media: *Bacillus subtilis* ATCC 6633 was grown on nutrient agar at 28°C for 24 h.

Nucleic acid preparation: Genomic DNA from *Bacillus subtilis* ATCC 6633 was extracted using DNP™ kit solution (Fermentas). Bacterial culture was centrifuged for 10 min at 7500 g and resuspended in 100 μL of protease buffer. It was then placed at 95°C for 10 min (before this step lysis solution pre-warmed by placing in 37°C for 20 min and gentle shaking). One hundred microliters of sample was mixed with 400 μL of lysis solution and vortexed for 15-20 sec. The sample should be completely homogenized at this step. Then 300 μL of precipitation solution was added and gently mixed by ten times inversion and placed in -20°C for 20 min, then centrifuged at 12,000 g for 10 min. After this step, the supernatant was decanted by gently inverting the tube and then placed on tissue paper for 2-3 sec. Subsequently, 1 mL washing buffer was added to pellet, gently mixed by inverting ten times and centrifuged at 12,000 g for 5 min, then decanted. The washing buffer was completely poured off and pellet dried at 65°C for 5 min. Then, the pellet was suspended in 50 μL of solvent buffer by gentle shaking and placing at 65°C for 5 min. In the final step, undissolved material was precipitated by 30 sec centrifuging at 12,000 g. Supernatant contains purified DNA.

Primers and PCR conditions: PCR reactions were carried out using forward primer (CGCGGATCCATGAAC AAAACAGAACTTATC) and reverse primer (TCCCCCGGGGTTTTCCGGCAACTGCGT) (Kohler and Marahiel, 1997) to amplify the *hbs* gene encoding HBsu protein in *B. subtilis*. PCR was carried out in a 50 μL volume containing 0.01 μg μL^{-1} template DNA, 0.5 μM of each primer, 5 μL of 10x *Taq Polymerase* buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.5 μL of Taq DNA polymerase and 31 μL of autoclaved distilled water. Amplifications were performed in a thermal cycler and initiated with a primary denaturation step at 93°C for 3 min, followed by 35 cycles of 93°C for 45 sec, 70°C for 30 sec and 72°C for 1.30 min. PCR products were analyzed by electrophoresis in 2% agarose gel and visualized by ethidium bromide staining. The product of amplification was a DNA fragment 290 bp in length and fragment sequencing was carried out from both ends using the same primer pair.

Probe preparation: Probe was labeled with DIG-11-dUTP (Roche, Manneheim, Germany) by PCR according to the manufacturer's instruction. For a standard PCR setting, the following nucleotide concentrations were used: 10 μM DIG-11-dUTP (Roch, Manneheim, Germany), 190 μM dTTP and 200 μM of each dATP, dGTP, dCTP. This concentration of labeled nucleotides allows the highly sensitive detection of PCR products after gel electrophoresis and Southern blotting.

To determine the probe yield, dilution series of labeled probe were spotted on a positively charged nylon membranes (Roche, Manneheim, Germany) and directly detected with DIG nucleic acid detection kit (Roche, Manneheim, Germany), using enzyme immunoassay and enzyme-catalyzed color reaction with NBT/BCIP.

Southern blot analysis: For Southern blot analysis, 0.5 μg μL^{-1} of genomic DNA from *B. subtilis* ATCC 6633 digested with *EcoRI* enzyme in a 20 μL reaction containing of buffer for 1 h at 37°C. The digested DNA was size-fractionated on 0.8% agarose gel containing ethidium bromide.

Upward capillary transfer of the DNA was performed over night onto positively charged nylon membranes using 0.4 N, followed by UV cross- linking.

The membrane was placed in a hybridization bag containing 20 mL prehybridization solution with 5×SSC, 0.1% (w/v) N-lauryl-sarcosine, 0.02 (w/v) SDS, 2% blocking reagent per 100 cm^2 of membrane surface area. The bag was sealed and prehybridized at the hybridization temperature, 68°C, for 1 h, then double-stranded DNA probes, heated in a boiling water bath for 10 min to denature the DNA, was chilled directly on ice. The probe was diluted in hybridization solution and at least 3.5 mL hybridization solution for a blot of 10×10 cm. The prehybridization solution was discarded from the bag and the hybridization solution containing the DIG-labeled probe was added. The probe was allowed to hybridize at 68°C over night. At the end of the hybridization, the hybridization solution was poured from the bag.

Detection of labeled probe: The membranes were washed for 2 min and blocked for 30 min using the DIG wash and block buffer set (Roche, Manneheim, Germany), followed by a new incubation for 30 min in blocking solution containing 0.075 U mL^{-1} Anti-Digoxigenin-Ab Fab fragments (Roche, Manneheim, Germany). Subsequently, the membranes were washed for 2×15 min in washing buffer and soaked in detection buffer for 3 min. Finally, the membrane was incubated in 10 mL freshly prepared color substrate solution in appropriate container in the dark for color development.

RESULTS AND DISCUSSION

The *Hbs* gene of the *B. subtilis* ATCC 6633 was successfully amplified and a single PCR product of the desired length, approximately 290 base pairs, was produced (Fig. 1).

Nucleotide sequence of the *hbs* gene was aligned with this gene in *B. subtilis* ATCC 23857 in the Gene bank and shown highconservation among the strains. In *B. subtilis* ATCC 6633, one nucleotide differs which does not bring about change in the codon (Fig. 2).

So, the PCR product was labeled using non-radioactive DIG-labeled nucleotide. The labeling of the PCR probes was analyzed through gel electrophoresis (Fig. 3).

For the selection of the specific yield of the labeling probe, dilution series of labeled PCR products was prepared and compared with DIG-labeled control DNA by DIG nucleic acid detection kit (Roche, Mannheim, Germany) by dot blotting (Fig. 4).

To verify the quality of the successfully amplified PCR probe (a 241 bp fragment of the chromosomal *hbs* gene), the labeled probe was hybridized to a chromosomal DNA blot and detected under conditions as indicated in materials and methods. Chromosomal DNA that had been cleaved with the restriction enzyme *EcoRI* revealed in the corresponding Southern blot the presence of one major band in each digest (Fig. 5). The probe recognized one band of about 5.4 kb in the *EcoRI* digests. This suggested the presence *hbs* gene within one these fragment.

More than 30 members of the family of histone-like proteins, all with a size of about 90 amino acids and an overall basic net charge, have been identified (Oberto *et al*., 1994). One of them is HU family. Several proteins with properties and structures highly homologous to those of HU protein have been isolated from bacteria and bacteriophages but it seems that HU protein sequence of various strains of one species could be different. Among some *Bacillus* species (*B. caldolyticus*, *B. globigii*, *B. stearothermophilus* and *B. subtilis*), a protein highly homologous to HU, classified HB, has been isolated and characterized (Wolfgang and Marahiel, 2002). Among the HB proteins of different *Bacillus* species so far sequenced, conservation is more than 80% (Kamaua *et al*., 2005). *Bacillus subtilis* genome encodes one HB protein by *hbs* gene that is called Hbsu (Kunst *et al*., 1997).

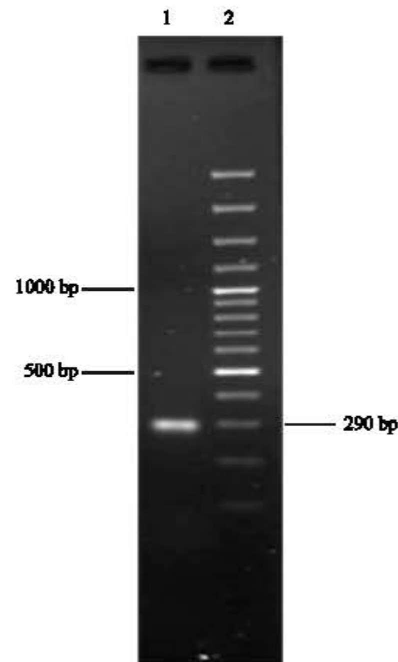

Fig. 1: Identification of the *hbs* gene of *B. subtilis* strains on 2% agarose gel. Lane 1: *hbs* gene, Lane 2: DNA size marker (100-3000 bp)

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                                                                                     57
B. subtilis ATCC 23857   ATG AAC AAA ACA GAA CTT ATC AAT GCG GTT GCA GAA GCA AGC GAA TTG TCT AAA AAA
B. subtilis ATCC 6633    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                                                                                    114
B. subtilis ATCC 23857   GAC GCT ACA AAA GCA GTT GAC TCT GTT TTT GAT ACG ATC TTA GAT GCA CTT AAA AAC
B. subtilis ATCC 6633    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                                                    171
B. subtilis ATCC 23857   GGT GAT AAA ATC CAA CTG ATC GGT TTT GGT AAC TTC GAG GTG CGT GAA CGT TCT GCA
B. subtilis ATCC 6633    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                                                                                    228
B. subtilis ATCC 23857   CGT AAA GGA CGC AAT CCT CAA ACA GGT GAA GAA ATC GAA ATT CCA GCA AGC AAA GTA
B. subtilis ATCC 6633    --- --- --- --- --C --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                                                                        276
B. subtilis ATCC 23857   CCT GCT TTC AAA CCA GGT AAA GCG CTT AAA GAC GCA GTT GCC GGA AAA
B. subtilis ATCC 6633    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
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Fig. 2: Alignment of the nucleotide sequences of the *hbs gene* from the *B. subtilis* ATCC 6633 with *B. subtilis* ATCC 23857 as reference

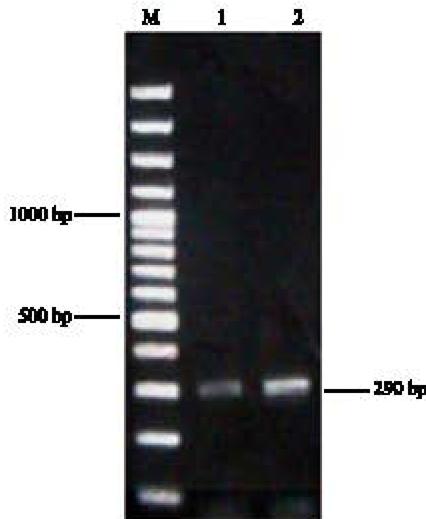

Fig. 3: Labeling of the PCR probes, Lane M: DNA size marker (100-3000 bp), Lane 1: Unlabeled PCR products, Lane 2: Labeled PCR products

Fig. 4: Dot blot direct detection for quantification of labeled probes and compared with DIG-labeled control DNA. Lane 1: Serial dilutions of DIG-labeled control DNA, Lane 2: Serial dilutions of the labeled PCR product

Detection of the *hbs* gene has been done before using a radioactive probe (Micka *et al*., 1991), but in this study, detection of the *hbs* gene was done using a specific non-radioactive method for probe labeling in *B. subtilis* strain. Thus, DNA from *B. subtilis* ATCC 6633 was extracted and *hbs* gene was successfully amplified. A

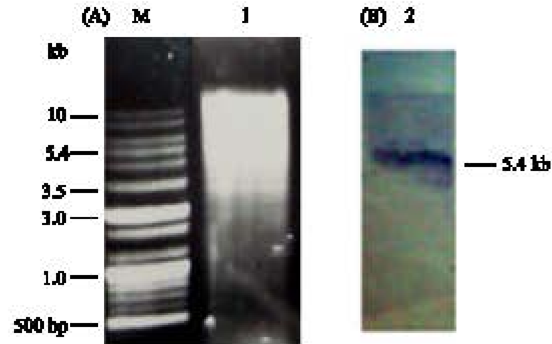

Fig. 5: Identification of the *hbs* gene of *B. subtilis*. (A) Lane M: DNA size marker (100-10000 bp), lane 1: Restriction analysis of genomic DNA isolated from *B. subtilis* ATCC 6633. Chromosomal DNA has been digested with the restriction enzyme *EcoRI*. The cleaved products were separated and visualized on a 0.8% agarose gel. (B) lane 2: Southern blot analysis revealed one major band after digestion by *EcoRI* restriction enzyme and hybridization with the PCR probe. The probe recognized one fragment at about 5.4 kb that has *hbs* gene

single PCR product of the length approximately 290 bp was produced. Then, PCR product was sequenced and shown to differ in just one nucleotide from *B. subtilis* ATCC 23857.

The PCR product from *B. subtilis* ATCC 6633 was labeled using a non-radioactive DIG-labeled nucleotide and the conditions for probe preparation and hybridization were optimized. Subsequently, the probe was examined in the detection of *hbs* gene in *Bacillus subtilis* ATCC 6633 by Southern blotting.

ACKNOWLEDGMENTS

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REFERENCES

Dixon-Fly, S.M. and L. Caro, 1999. Characterization *in vitro* and *in vivo* of new HU family protean from *Streptococcus thermopiles* ST11. Plasmid, 42: 159-173.

Kamaua, E., N.D. Tsihlis, L.A. Simmons and A. Grove, 2005. Surface salt bridges modulate the DNA site size of bacterial histone-like HU proteins. Biochem. J., 390: 49-55.

Kohler, P. and M.A. Marahiel, 1997. Association of the his tone-like protein hubs with the nucleotide of *Bacillus subtitles*. J. Bacteriol., 179: 2060-2064.

Kunst, F., N. Ogasawara, I. Moszer, A.M. Albertini and G. Alloni *et al*., 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature, 390: 249-256.

Micka, B., N. Groch, U. Heinemann and M.A. Marahiel, 1991. Molecular cloning, nucleotide sequence and characterization of the *Bacillus subtilis* gene encoding the DNA-binding protein HBsu. J. Bacteriol., 173: 3191-3198.

Micka, B. and M.A. Marahiel, 1992. The DNA-binding protein Hubs is essential for normal growth and development in *Bacillus subtitles*. Biochimie, 74: 641-650.

Oberto, J., K. Drlica and J. Rouviere-Yaniv, 1994. Histones? HMG? HU? IHF: Meme combat. Biochimie, 76: 901-908.

Ross, M.A. and P. Setlow, 2000. The *Bacillus subtilis* HBsu protein modifies the effects of alpha/beta-type, small acid-soluble spore proteins on DNA. J. Bacteriol, 182: 1942-1948.

Schmid, M.B., 1990. More than just histone-like proteins. Cell, 63: 451-453.

Swinger, K.K. and P.A. Rice, 2007. Structure-based analysis of HU-DNA binding. J. Mol. Biol., 365: 1005-1016.

Ussery, D., T. Schou Larsen, K.T. Wilke, C. Friis, P. Worning, A. Krogh and S. Brunak, 2001. Genome organization and chromatin structure in *Escherichia coli*. Biochimie, 83: 201-212.

Wolfgang, K. and M.A. Marahiel, 2002. Structure-function relationship and regulation of two *Bacillus subtilis* DNA-binding proteins, HBsu and AbrB. J. Mol. Microbiol. Biotechnol., 4: 323-329.

Yamane, K., K. Bunai and H. Kakeshita, 2004. Protein traffic for secretion and related machinery of *Bacillus subtilis* Biosci. Biotechnol. Biochem., 68: 2007-2023.

Yokohama, E., K. Doi and S. Ogata, 1997. Cloning and sequencing of the *hup* gene encoding the histone-like protein HSl of *Streptomyces lividans*. Biochim. Biophys. Acta, 1353: 103-106.