

Optimization of RAPD-PCR for discrimination of different strains of *Bacillus thuringiensis*

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Abstract

A randomly amplified polymorphic DNA fingerprinting assay has been optimized that discriminate different B. thuringiensis isolates and serotypes. Due to advance in molecular biology technique, large numbers of highly informative DNA markers have been developed for the identification of genetic polymorphism. In the last decade, RAPD technique has been one of the most commonly used PCR based molecular markers. Relationships between species may be determined by comparing their unique fingerprint information, which are expected to be identical among related species. The optimization of certain parameters like PCR buffer, magnesium concentration and suitable primer, which produces discriminatory and reproducible fingerprints of B. thuringiensis isolates, was achieved. In addition an alkaline lysate method was optimized which give reproducible results.

Keywords: Bt., Whole cell, RAPD, PCR

Introduction

Random amplification of polymorphic DNA (RAPD) is a modification of the polymerase chain reaction (PCR) in which a single primer able to anneal and prime at multiple location throughout the genome can produce a spectrum of amplification products that are characteristics of the template DNA J. Welsh & JGK Williams[1,2]. RAPD markers have found a wide range of application in gene mapping, population genetics, molecular evolutionary genetics and plant and animal breeding. This is mainly due to the speed, cost and efficiency of the RAPD technique to generate large numbers of markers in a short period compared with previous methods. Therefore, RAPD technique can be performed in a moderate laboratory for most of its applications. It also has the advantage that no prior knowledge of the genome under research is necessary [3].

Typing methods must be reliably able to recognize related strains and to discriminate between unrelated strains. However, the reproducibility of RAPD profiles generated from bacterial templates has caused researchers apprehensive when using RAPD for genome fingerprinting [4]. Optimization of the RAPD reaction and thermal cycling protocol can eliminate most of variation that are sometimes observed in duplicate DNA profile [5], but other parameters which is important is DNA polymerases from different companies gave dissimilar results, thermal cyclers of different company with different rapping effect has also effect the reproducibility of results [6]. In addition, a technique to obtain reproducible RAPD

fingerprints of *Bacillus thuringiensis* isolates without the need to purify genomic DNA is described.

Material and Method

Bacillus thuringiensis were isolated by sodium acetate selection method as described by Tavera et al. [7] from the soil samples collected from different districts of Vidharbha, Maharashtra, India. All the isolates were also tested for cry gene amplification as well as different biochemical characteristics described by Sneath [8] and morphological characterization of crystal protein by staining method described by Smirnhoff [11]. SEM studies of different crystal proteins were done by preparing sample as describe by Mitchell [9]. A 50 µl of Bacterial sample was smeared on carbon tape and fixed by immersion in 3% glutaraldehyde with 0.1M sodium cacodylate buffer glutaraldehyde with 0.1 M sodium cacodylate buffer overnight and were washed with 0.1 M sodium cacodylate three times. This was followed by post-fixing in 1% OsO₄ in the same buffer for 3 h and rinsing with the same buffer and deionized water three times for each treatment. The samples were dehydrated by an ethanol series of 40, 50, 60, 70, 80, 85, 90, 95, and 100% ethanol and stored in 100% ethanol. Specimens in 100% ethanol were critical-point dried in a vacuum drier and mounted on aluminum stubs and gold-coated for 5 min and observed under SEM (Analytical scanning electron microscope; JEOL, JSM-63804).

Preparation of template DNA for PCR

DNA was extracted using modified protocol of Rosso and Delecluse [10]. 1.5ml of 18-20h old Luria-Bertani broth cultures were centrifuged at 3,000× g for 5 min at 4°C, and the pellets were washed again in 100µl of J buffer (1.0 M Tris-HCl, 0.1 M EDTA, 0.15 M NaCl [pH 8]). Pellets were resuspended in 100µl of J buffer, and lysozyme was added to a final concentration of 4 mg/ml, followed by incubation at 37°C for 30 min. 1 µl of RNase (10 mg/ml) was added, and suspensions were incubated for 30 min followed by the inactivation of the enzyme at 65°C for 10min. 20 µl of 20% sodium dodecyl sulfate was added to the mixture and incubated for 20 min at 70°C, followed by the addition of 2 µl of proteinase K (10 mg/ml) and incubation overnight at 55°C. A total of 1.15 ml of NaCl 6 M was then added, gently mixed in ice for 15 min, and centrifuged at 3,900 × g for 20 min at 4°C. The supernatant was mixed with an equal volume of isopropanol and centrifuged at 17,000 × g for 20 min at 4°C. The pellet was washed with 70% ethanol, air dried, and dissolved in 50 µl of Tris-EDTA buffer (pH 8). DNA was quantified by spectrophotometry, and samples were stored at -20°C until further use.

Preparation of template by whole cell method

Colonies obtained on sporulating medium were directly used for the preparation of whole cell suspension. A well defined colony was suspended to 100µl of 0.05M NaOH and incubated at 95°C for 15 min and centrifuged at 14000 rpm for 2 min. The suspension was preserved at 4°C.

Preparation of RAPD reaction

Thirty primers were used in this study is synthesized by Integrated DNA Technology, Coralville, USA. All the primers were first used to check the polymorphism with the purified DNA as well as DNA obtained from whole cell. PCR amplification were performed with 25 µl reaction volume containing 20mM Tris-Cl pH-8.8, 10mM KCl, 10mM Ammonium sulphate, 2mM MgCl₂, 0.1% TritonX100 and 1% DMSO. Amplification conditions used for RAPD PCR were an initial denaturation at 94°C for 6 min., followed by 40 PCR cycles at 94°C for 45sec, a primer annealing at 36°C for 1 min, and primer extension at 72°C for 1 min. A final extension of 10 min at 72°C was carried out for polishing the ends of PCR products.

For the visualization of amplified products the samples were analyzed by agarose gel electrophoresis as per the procedure described by [11]. The amplified products of RAPD PCR along with the DNA molecular weight marker (Low range DNA ruler or 100bp and 1 kb DNA Ladder) were separated on 1.5% agarose gel containing 0.5 μ g/ml of ethidium bromide in 1X TBE at 50 volts for approximately 3 hrs. The documentation was done with the help of AlphaDigiDoc 1201 system.

Results

Isolation and Characterization of crystal protein

Preliminary identification were made by growing isolates on T3 media and staining was done by the method describe by Smirnoff, W. A. [12]. The crystal protein appears violet under light microscope 100x (Fig.1A) The presence of crystal protein was further confirmed by scanning electron microscopy (SEM) under higher magnification for better visualization of the shape (Fig.1B).

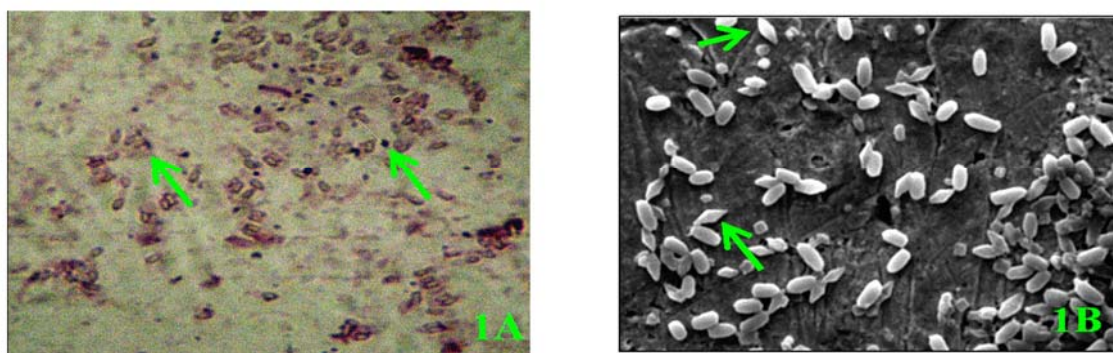


Figure 1A & 1B. Detection of crystal protein by staining and scanning electron microscopy
1A: Crystal protein stained violet to dark blue and spores appear pink; **1B:** Bipyramidal structure of crystal protein (indicated by arrow) evident from the Scanning electron

Effects of (MgCl₂) on RAPD

Less than 2mM conc of MgCl₂ reduces the yield of amplification products. Increasing concentration from 2 to 3.5mM did not give any difference in yield of amplicons where as from these observations a conc. of 2mM MgCl₂ can be considered as the optimum conc. for RAPD of Bt.

Effect of template DNA on RAPD

Amplification was performed using Primer OPD-18 with dilution of purified DNA as template. DNA conc in range of 20 ng gave optimal amplification (Fig.2A) while higher concentration shows no improvement in amplification and shows smear background (Fig. 2B)

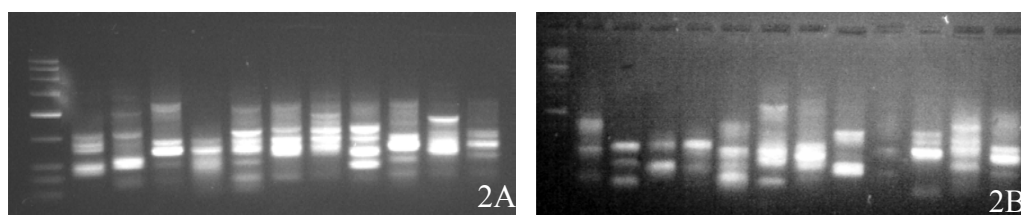


Figure 2. Effect of template DNA concentration on PCR amplification. 2A: PCR amplified product obtained with Optimal DNA concentration of 20 ng; 2B: PCR amplified product obtained with higher DNA concentration.

Effect of DMSO and comparison of profile generated using phenol extracted DNA and whole cell template

Without using DMSO we get reproducible results (Fig.3) Lane 3, 6, 9, 12: sample without DMSO containing whole cell template of *B. thuringiensis* with different serotypes.

Selection of primers

Along the 30 screened primers some of the primer with GC% 70 was found to be produced clear and reproducible amplicons. Primer like OPA-1, OPA-11, OPA-19 & 13 produced discrimination pattern as well as it shows very similar amplification pattern between isolates (Fig.4) having same serology and strains showing toxicity to *Bombyx mori*. Primer OPA-2, OPA-3 produce less bands while OPH series produces maximum amplicons. RAPD analysis was able to distinguish *B. thuringiensis* subsp. *kurstaki* H3abc from the three *B. cereus* strains tested. OPA-1 discriminate all the serotypes with reproducible results (Fig.5)

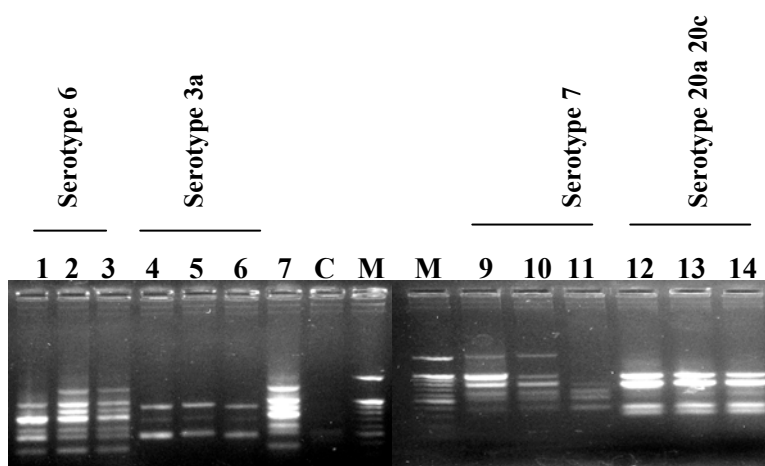


Figure 3. RAPD profile of Serotype 6, 3a, 7 & 20a20c with varying reaction parameters. Lane 1, 4, 7, 10: purified DNA without DMSO; Lane 2, 5, 8, 11: Lysate with DMSO; Lane 3, 6, 9, 12: Lysate with DMSO; M: DNA Molecular Marker; 7: Repeation of Lane 2; C2: Negative Control.

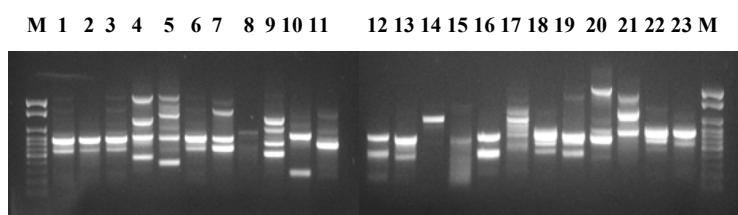


Figure 4. RAPD profile of different serotype local isolates and different strains of *Bacillus sp.*

- Lane 1,2,3,22,23 represent serotype 3a 3b 3c
- Lane: 4, 5,21 represent *Bacillus cereus*
- Lane: 6 Bt serotype 3a 3b
- Lane: 7 serotype H14
- Lane : 11, 12, 13, 14 *Bacillus megaterium*

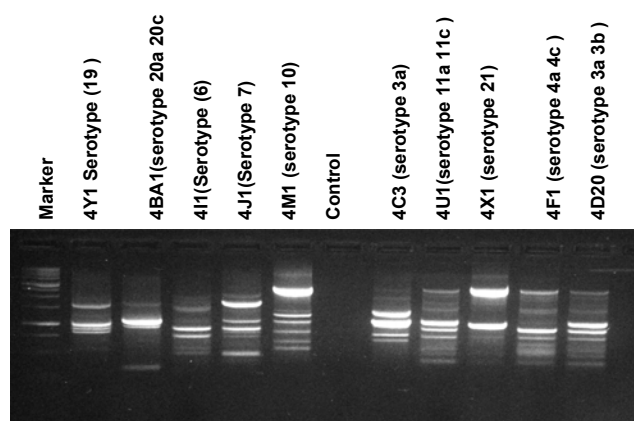


Figure 5. Amplified product of different Serotypes showing discrimination using OPA-1 random primer

Discussion

In this study random primers were used to amplify genomic DNA from reference as well as isolates using a protocol optimized in terms of reaction buffer ($MgCl_2$), template concentration template preparation method and choice of primers. The reaction mixture contains 20-50ng of genomic DNA, 0.2mM each dNTP, 20 moles of RAPD primer, 0.5U of Taq DNA polymerase with buffer containing 20mM Tris-Cl pH-8.8, 10mM KCl, 10mM Ammonium sulphate, 2mM $MgCl_2$, 0.1% TritonX100 and 1% DMSO. Amplification was done with a reaction volume of 25 μ l in My Cycler (Biorad) with an initial denaturation at 94°C for 6 min., followed by 40 PCR cycles at 94°C for 45sec, a primer annealing at 36°C for 1 min, and primer extension at 72°C for 1 min. A final extension of 10 min at 72°C was carried out for polishing the ends of PCR products. Cations such as K^+ or NH_4^+ affect PCR specificity by binding to the negative charged phosphate groups on DNA, thus facilitate primer/ template annealing. The yield and specificity of the PCR can thereby be increased by altering the concentration of KCl and $MgCl_2$. $MgCl_2$ stabilizes primer annealing; therefore, the concentration of $MgCl_2$ have a large effect on the specificity and yield of a reaction [13]. Too little Mg^{2+} can decrease the yield while excess Mg^{2+} results in non-specific amplification as a result of reduced enzyme fidelity. In contrast to these findings, [14] produced identical profiles when three reaction buffers differing in composition were interchanged using the same brand of Taq DNA polymerase. Sensitivity to small changes in pH at certain $MgCl_2$ and KCl concentration led to highlight the importance of accurate pH measurement during reaction buffer preparation [15]. However, preparation of master mix, pipetting error and proper handling may increase reproducibility.

The ratio of DNA template to primer is one of the most critical factors to consider when optimizing the PCR; therefore, a range of DNA concentration should be tittered against a fixed primer concentration for each DNA extraction protocol to obtain the ideal conditions [16]. Although it has been suggested that phenol-extracted DNA can aid reproducibility, using whole cell template may also give reproducible result with proper optimization of DNA concentration this method leads to save time during investigation of large sample. However, comparison of phenol-extracted DNA template profiles and boiled whole cell template profiles by [17] revealed that profiles were usually similar but some differences occurred as a result of damage to large DNA fragments during boiling. This may also lead to a lack of reproducibility as damage to the DNA is random event [18] found that ethanol-treated whole

cell template gave profiles almost identical to those obtained from phenol-extracted DNA. However, reaction products were less stable and profiles smeared if not analysed within 24 h post amplification. We have found lysate method by NaOH gave more stable as well as reproducible results up to fourth days of its amplification. [19] Suggested that, due to the distribution of binding sites, certain primers may amplify regions of the genome that overlap and, therefore, amplify from only a small section of the genome, thus possibility not reflecting the true heterogeneity between strains. Other primers may amplify different parts of the genome and so different between strains, it is therefore, important to try more than one primer in RAPD analysis. In this study we have used 1% DMSO which in final concentration in reaction. BSA in concentration of upto 0.8% ug/ul appeared to increase the efficiency of the PCR reaction much more than either DMSO or glycerol. In our study reproducible results were obtained without DMSO in both purified as well as whole cell lyses method but as our buffer contain .1% triton-X 100 this may lead to increase the specificity of primer [20]. RAPD is found to be more beneficial for discriminating different serotype with more clearly reproducible results if proper template DNA isolation method optimization of reaction buffer is used and second thing is choice of proper primer, which gave polymorphic or highly discriminating results

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