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Screening and Identification of PGA Producing *E. coli* Isolates by PCR Technique

[1]Kafshnochi Magsoud, [2,3]Safar Farajnia, [1]Raheb Aboshof and [2]Hanieh Rezaee
[1]Department of Microbiology, Faculty of Biology,
University of Baku State, Baku, Azerbaijan Republique
[2]Drug Applied Research Center, [3]Biotechnology Research Center,
Tabriz University of Medical Sciences, Tabriz, Iran

Abstract: The PGA gene of *Escherichia coli* encodes a penicillin G acylase (PGA). Penicillin G acylase 1(PGA, EC 3.5.1.11) is a type II penicillin acylase that hydrolyzes Penicillin G to 6-aminopenicillanic acid (6-APA) and phenyl acetic acid (PAA). PGAs have been found in numerous bacteria and fungi and the PGA of *Escherichia coli* has been well characterized. In free-living *E. coli*, PGA is thought to act as a Scavenger enzyme for many different natural esters and amides of PAA and its derivatives, such as hydroxyphenylacetic acid (HPA). In this study *E. coli*, a member of Enterobacteriasea was investigated for PGA. The main aim of this study was screening of *E. coli* strains from environmental and clinical specimens for PGA by PCR and then cloning, sequencing and recombinant expression of cloned PGA in the *E. coli*. Total 280 *E. coli* isolates were collected from water, soil and clinical specimens. Specimens were transported to the laboratory and then routine tests were done for identification. In this study we found PGA gene in only 6 *E. coli* isolates. All of PGA positive isolates were from Clinical specimens. The PGA gene from one of the positive isolate were amplified and cloned in pGEM T-easy vector. This clone will be used for further study and production of recombinant Penicillin acylase.

Key words: PGA, PCR, *E. coli*, screening and indentification

INTRODUCTION

Enzymatic catalysis avoids the need for environmentally harmful chemicals and can reduce the waste treatment costs. Furthermore, the reactions can be carried out in mild conditions, leading to increased yield by reducing side-products. As regards the preparation of pure enantiomers, the enzymatic reactions minimize problems of isomerization, racemization, epimerization and rearrangement that may occur during chemical processes. *E. coli* PGA that is induced by PAA in the original *E. coli* strain can be produced constitutively by an *E. coli* transformant that harbors a plasmid containing the PGA gene (Mayer *et al*., 1979).

Penicillin acylases are classified into three groups based on substrate specificity: PGA, penicillin V acylases (PVA) and Ampicillin acylases (Valle *et al*., 1991). PGAs catalyze the cleavage of the acyl chain of Penicillins to yield 6-aminopenicillanic acid (6-APA) and the corresponding organic acid (Fig. 1).

The expression regulation of PGA is considered to be very complex and even unique (Ile *et al*., 1991). Many

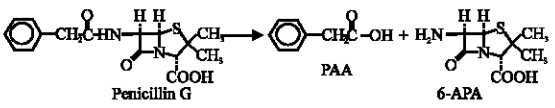

Fig. 1: Reaction scheme of penicillin G hydrolysis. The substrate is converted into phenyl acetic acid (PAA) and 6-aminopenicillanic acid (6-APA)

factors, such as temperature, PAA, O2 levels and even strain variations may affect the expression of PGA. It has been demonstrated that PAA induction is involved in PGA transcription, but the PAA regulatory mechanism is not well clear (Horton, 1993). The expression of PGA was regulated in a similar manner in both *E. coli* ATCC1105, which originally produces PGA and the PGA -clone transformed *E. coli* HB101. Therefore, it is assumed that in both strains tested, common regulatory proteins were involved in PAA induction. In addition to its assumed auto-regulatory character, temperature, PAA and variation of *E. coli* strains may also affect the PGA repressor transcription.

Corresponding Author: Magsoud Kafshnochi, Department of Microbiology, Faculty of Biology,
University of Baku State, Baku, Azerbaijan

The routine method for PGA production is from the *E. coli* ATCC1105. But this method produce very low level of PGA and have limitations, like existence of PAA in the culture permanently that it is very expensive. It seems that production by recombinant protein technology can have PGA enzyme more and cheap. Betalactame antibiotics, penicillin and cephalosporin are produced by fermentation reactions and penicillium or cephalosporium fungi (Robas and Branlant, 1994). Pen G, Pen V and Cef C are the primary substrates for generation of penicillin and cephalosporin antibiotics groups. Also, acyle group in the 6-amino position of penicillin and acyle group in the 7-amino of cephalosporin antibiotics include side chain of molecule. The side chains of Pen G, Pen V and Cef C are Phenyl acetyl, Phenoxy acetyl and Amino adipyl. This side chains were deleted by amid bond fracture form 6APA (6- Amino penicillanic acid) for Pen G, V and 7-ACA (7- Amino cephalosporanic acid) for cephalosporin (Virden, 1990).

In the base of the substrate kind and molecular organization, acylases divided into many groups. Substrate specification was fixed by side chain of enzyme that recognizes side chains of Betalactame molecules (Schumacher *et al*., 1986). PGA is used for hydrolyze of Pen G, Cef g to 6-APA and 7-ADA that they are the substrates for generation of semi-synthetic penicillins or cephalosporins (Sudhakaran *et al*., 1992). The semi-synthetic penicillins or cephalosporins have resistance to betalactamase and so have the benefits more than primary molecules. In this study PCR technique was used for screening of *E. coli* isolates to identify PGA producing *E. coli* strains.

MATERIALS AND METHODS

Chemicals, enzymes and strains: Restriction endonuclease, T4 DNA ligase and Taq DNA polymerase were obtained from Fermantas (Lithuania) and were used as recommended by the manufacturer. *E. coli* DH5-a (Invitrogen, Netherlands) were used as host strains for the cloning experiments. The plasmid pGEM-T (Promega, USA) was used for cloning, experiment.

Strains and culture media: *E. coli* strains were isolated from environmental and clinical specimens. To obtain isolates of *E. coli*, specimens were streaked onto eosin methylene blue agar (Difco) plates. Those which developed a characteristic metallic sheen were selected and confirmed to be *E. coli* by biochemical tests. Only 1 isolate was selected from each sample and a total of 280 isolates were collected. The organisms were maintained on nutrient agar (Difco) slants at 4°C until they were used.

Extraction of DNA from bacterial cultures: The Luria-Bertani (LB) broth [composed of yeast extract 1.0% (weight/volume, w/v), NaCl 0.5% (w/v) and tryptone 1.0% (w/v)] was inoculated with bacterial isolates and incubation was done at 37°C for 24 h. After centrifugation of 3 mL of culture at 5000 rpm for 1 min, the supernatant was removed and the pellet was resuspended in 360 μL of TE buffer (Tris 10mM, pH 8.0 and EDTA 1mM) containing 10 μg mL^{-1} proteinase K and kept at 37°C for 30 min. It was followed by the addition of 1% SDS and incubation at 65°C for 1 h. DNA was extracted by conventional phenol/chloroform method and followed by RNase treatment for the removal of contaminating RNA. The purity of extracted DNA was checked by measurement of A260 and A280 and agarose gel electrophoresis.

Table 1: The sequences of primers were used for amplification and nested PCR confirmation of PGA gene

Full orf primers	PGA F1	(5'- ATGAAAAATAGAAATCGTATGATC- 3')
	PGAR1	(5'- TCTCTGAACGTGCAACACTTC-3')
Nested primers	PGA F2	(5'- TGTCGCGATGATATTTGTG- 3')
	PGAR2	(5'- GCGGGATTTAGCGTAAGC-3')

PCR amplification of PGA gene from *E. coli* isolates: The genomic DNA isolated from bacterial isolates was entered in PCR reactions containing primers complementary to PGA sequence. Primers (Table 1) were designed based on PGA gene sequences reported from *E. coli* ATCC 11105 (Gene Bank Accession Numbers P06875). An Eppendorf DNA thermal cycler and Taq DNA polymerase, (Fermentas, Lithuania) was used for PCR amplification of desired fragment. The reaction mixture included 0.4 μM of each primer, 200 μM dNTPs and 2 mM $MgCl_2$ PCR conditions were: primary denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 5 min.

Cloning and DNA sequencing: The PCR product from positive isolates were entered in a ligation reaction with pGEM-T easy vector and transformed into the *E. coli* DH5a cloning host strain. Clones were screened on LB agar containing ampicillin (100 mg mL^{-1}), X-Gal (1mM) and IPTG (1 mM) and white colonies were selected for plasmid isolation. Positive clones were selected by PCR and confirmed by nested PCR and restrict map analysis. One positive clone were submitted for sequencing with di-deoxy chain termination method.

DNA and predicted amino acid sequences were analyzed and compared with sequences present in the database using Blast program of NCBI. The World Wide Web Prediction Server SignalP V1.1was used to determine the presence and locations of signal peptide cleavage sites in amino acid sequences.

RESULTS AND DISCUSSION

In the present study, the PCR technique was used for screening and identification of penicillin G acylase enzyme

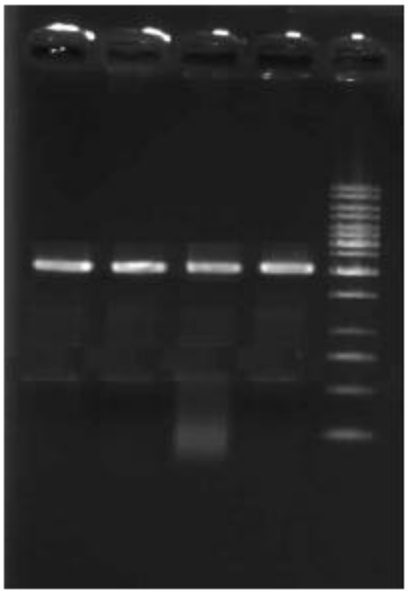

Fig. 2: PCR amplification og PGA gene from an *E. coli* isolates

producing *E. coli* strains isolated from clinical and environmental samples. The PCR technique allows the production of more than 10 million copies of a target DNA sequence from only a few molecules in a few hours. The sensitivity of this technique provided a valuable tool for rapid and accurate method for detection of PGA producing strains.

In this study, Screening of *E. coli* isolates for PGA gene resulted into the identification of 6 PGA producing *E. coli* strains among 280 screened isolated. The gene from one positive isolates (Fig. 2) was cloned in pGEM-Teasy vector and used for DNA sequencing. Sequence analysis showed the gene composed of 2538 nucleotide encoding 846 amino acids. Blast analysis of obtained sequence showed that the gene contain 98% homology to previously reported PGA gene from *E. coli* strains. The amount of homology to other reported PGA genes were: 96% to *Achromobacter xylosoxidans* and 81% homology to *Klevromyces citrophila*.

Analysis of predicted amino acid sequence by Server SignalP program revealed the presence of a signal peptide of 29 amino acids in the N terminal region of coded protein. The results of our study show that the PGA gene is highly conserved among *E. coli* strains. The high conservation of PGA gene in different bacteria may implicate importance of this gene for these bacteria.

CONCLUSION

This study showed the applicability and advantages of PCR technique for rapid detection of Penicillin G Acylase harboring wild *E. coli* isolates from different sources. The results of our study could expand the application of PCR as a cheap and high speed method for screening of isolates with pharmaceutical and biological important characteristics.

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