African Journal of Biotechnology Vol. 9(18), pp. 2697-2701, 3 May, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Cloning and over-expression of Penicillin G acylase in *Escherichia coli* BL21

Magsoud Kafshnochi[1,3], Safar Farajnia[2,4]*, Raheb Aboshof[3], Hossein Babaei[4] and Mona Aminolroayaee[4]

[1]Department of Microbiology, Bonab Islamic Azad University, Bonab, Iran.
[2]Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
[3]Department of Microbiology, Faculty of Biology, Baku State University, Azerbaijan Republic.
[4]Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

Accepted 26 February, 2010

Penicillin G acylase (PGA) is one of the most important enzymes in the pharmaceutical industry. It is utilized in the process for production of semi-synthetic penicillins. Several different penicillin acylases with various characteristic have been isolated from different bacteria and identification of bacterial isolates harboring PGA enzyme with higher industrial compatibilities is of high interest. The aim of this study is to screen for PGA producing *Escherichia coli* isolates as well as the cloning and recombinant expression of PGA for high level enzyme production. Bacteria isolated from environmental and clinical samples were identified by standard microbiological tests and then *E. coli* isolates were subjected to DNA extraction and PCR screening using primers designed on conserved region of PGA genes. The PCR product from a positive isolate were cloned and subjected to sequencing. The gene encoding for full length PGA was expressed in *E. coli* under the T7 promoter. PCR screening identified several PGA positive *E. coli*. One of the positive isolates was cloned in pGEM -T easy vector. Sequencing of the cloned gene revealed that the gene encoding Penicillin G acylase from this wild *E. coli* isolate contains an open reading frame of 2538 nucleotide encoding 846 amino acids. Analysis of the sequencing results showed that the PGA gene is highly conserved among *E. coli* strains. Recombinant expression of PGA from wild isolate in *E. coli* BL 21 resulted in a high level expression of recombinant PGA which appeared as a dense band in SDS-PAGE analysis of induced culture.

Key words: PGA, cloning, sequencing, recombinant expression.

INTRODUCTION

Traditionally, semi-synthetic penicillins were produced by chemical synthesis that needs a series of complex chemical reactions at low temperatures, using toxic compounds. These undesirable solvents need to be removed and there is a need for a rigid quality control of the downstream purification steps. In recent years, chemical synthesis has mostly been replaced by less polluting biological methods using enzymes isolated from different microorganisms (Shewale et al., 1990; Demain, 2000). These enzymatic reactions work in aqueous medium and physiological conditions without producing toxic wastes (Van Langen et al., 2001).

Penicillin G acylase (PGA) is a type II penicillin acylase that hydrolyzes Penicillin G to 6-aminopenicillanic acid (6-APA) and phenyl acetic acid (PAA) (Ohashi et al., 1989; Zietkiewicz et al., 1994). 6-APA is starting material for production of semi-synthetic penicillins (Kumar et al., 2007; Ochman et al., 1988). PGA has been found in numerous bacteria and the PGA of *Escherichia coli* has been extensively studied (Parmar et al., 2000).

Although the exact function of PGA in free-living *E. coli* have not been well understood, it is thought to act as a

*Corresponding author. E-mail: farajnias@tbzmed.ac.ir. Tel: 0098301 8589. Fax: 0098411-3363231.

Abbreviations: **PGA,** Penicillin G acylase; **6-APA,** aminopenicillanic acid; **PAA,** phenyl acetic acid; **EMB,** eosin-methylene blue; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **PCR,** polymerase chain reaction; **IPTG,** isopropyl--D-thiogalactopyranoside; **BLAST**, basic local alignment search tool; **PDAB,** p-dimethyl-aminobenzaldehyde.

scavenger enzyme for various natural esters and amides of PAA and its derivatives, such as hydroxyphenylacetic acid (Díaz et al., 2001). Thus, when *E. coli* encounters phenylacetylated compounds, the periplasmic PGA degrades them to PAA and this moiety then diffuses into the nucleoplasm.

The characteristics of PGA isolated from different biological and environmental sources were found to be varied in different aspects including, substrate specificity, optimum pH, temperature tolerance etc. Therefore, microorganisms have been extensively screened for isolation of novel penicillin acylases with higher compatibility with industrial deacylation requirements. In recent years, recombinant DNA technology has emerged as a potent technology for high level production of many useful proteins. In this method, desired and increased yield are achieved by proper vector and host cell selection. Production of PGA by recombinant technology could have several advantages. Firstly, recombinant strain could express higher level of recombinant protein compared to native strains. Secondly, the expression of PGA in native *E. coli* is under a complex control circus (Wang et al., 2004), whereas in recombinant *E. coli* the expression could be under tight control. The objective of this study was to screen *E. coli* isolates from clinical and environmental samples for PGA. The gene from a positive clone was cloned and the T7 promoter and BL21 host cell were investigated for high level production of active PGA.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids

The *E. coli* strains, DH5α (Invitrogen, Carlsbad, CA) and BL21 (DE3) (Novagen, USA) were used for cloning and protein expression, respectively. Strains were cultured at 37°C in Luria-Bertani (LB) broth. Ampicillin was used at a concentration of 100 $\mu g ml^{-1}$ for selecting transformed bacteria. The plasmid pGEM-T Easy vector (Promega) was used for cloning of the amplified Penicillin G acylase gene (PGA) and the plasmid pET 22 b was used for protein expression.

Isolation of *E. coli* from samples

In this study, *E. coli* strains isolated from water, soil and clinical specimens were screened for PGA. A total of 280 specimens were collected from water, soil and clinical specimens. Samples were transported to the laboratory and then standard microbiological tests including culture on eosin-methylene blue (EMB) agar, gram staining and biochemical tests like Oxidase, Indole, Voges-Proskauer (VP), methyl red (MR) and Citrate tests (IMVIC) (Forbes et al., 2006) were carried out for identification of isolates. Only 1 isolate was selected from each sample. The organisms were maintained on nutrient agar (Difco) slants at 4°C until they were used.

DNA extraction and PCR screening

Isolates were cultured in LB broth overnight at 37°C with vigorous shaking and the bacteria were pelleted by centrifugation at 9000 rpm. DNA isolation was carried out essentially as described (Montazam et al., 2009) with slight modification. Briefly, the bacterial pellet was resuspended in TE buffer (Tris 10 mM, PH 8, EDTA 1 mM) containing SDS (1%) and proteinase K (10 mg/ml) and incubated at 55°C for 2 h. After that, an equal volume of phenol-chloroform (1: 1) was added, incubated at room temperature for 5 min and centrifuged for 5 min at 10000 rpm. The aqueous phase was transferred into a new tube, incubated with RNAse A (10 mg/ml) for 20 min at 37°C and then DNA was precipitated by adding 2.5 volume of 100% cold ethanol. The pellet was then washed with 70% ethanol and dissolved in distilled water after drying. DNA extracted from *E. coli* isolates were subjected to PCR amplification with primer pair PGA F1, 5'- TGTCGCGATGA TATTTGTG- 3' and PGA R1, 5'- GCGGGATTT AGCGTAAGC-3'. Primers were selected based on conserved regions of PGA genes that have already been reported. PCR amplification was carried out in a final volume of 25 µl containing 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 18 mM NaCl, a 0.2 mM concentration of each 2′-deoxynucleoside triphosphate (2′-dNTP) (dATP, dCTP, dTTP and dGTP), a 0.4 µM concentration of each forward and reverse primer and 1 U of *Taq* DNA polymerase (Fermentas, Litany). Thermocycler conditions for PCR were one cycle of 94°C for 4 min, 32 cycles of 94°C for 45 s, 50°C for 30 s and 72°C for 60 s and one cycle of 72°C for 5 min followed by a hold at 10°C. The PCR results were assessed by electrophoresis on 1% agarose gel and visualized by staining with ethidium bromide (EtBr).

Cloning and sequencing of full length PGA gene

After identification of PGA producing strains, PCR amplification was performed on a positive strain using primers F2, 5'-TGGCCATGA AAAATAGAAATCGTATGATC-3' and R2, 5'-GCTCGAGTCTCTGA ACGTGCAACACTTC-3', which were complementary to 5' and 3' end of full length PGA gene, respectively. The PCR product was purified with a PCR purification kit (Qiagen) according to the manufacturer's instructions and used for T-A cloning using pGEM-T easy cloning kit (Promega) which yielded plasmid clone pGEM-PGA. The reaction was transformed into an *E. coli*, DH5a competent cells and a positive clone was submitted for sequencing with M13 forward and reverse primers. Nucleotide and predicted amino acid sequences were compared to data available by the BLAST search method.

Recombinant expression of PGA in *E. coli*

The coding region for full length PGA gene was isolated by digestion of pGEM-PGA clones with *Msc* I-*Xho* I restriction enzymes and subcloned into the *Msc* I-*Xho* I site of the pET 22b expression vector (Novagen) in frame with a carboxy-terminal six histidine tag, yielding the plasmid subclone pET22b-PGA. Sequencing with vector-based primers demonstrated that the His-tag fusion was in frame. The construct was transformed into *E. coli* BL21strain, cultured at 37°C in LB medium containing 100 µg ml^{-1} ampicillin to an absorbance at 600 nm (A_{600}) of 0.7 unit. Protein expression was induced by adding isopropyl--D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and analyzed by SDS-PAGE.

Biologic assay for PGA production by recombinant Bacteria

E. coli BL21 containing pET 22b-PGA construct was cultured in media I (1% Tripton, 0.3% Beef extract and 0.15 % Phenyl acetic acid) to an absorbance of 0.8 and induced by addition of IPTG (1 mM). After two hours, the cells were collected by centrifugation and PGA production was estimated by the p-dimethyl-aminobenz-aldehyde (PDAB) method (Shewale et al., 1987). Briefly, the cells

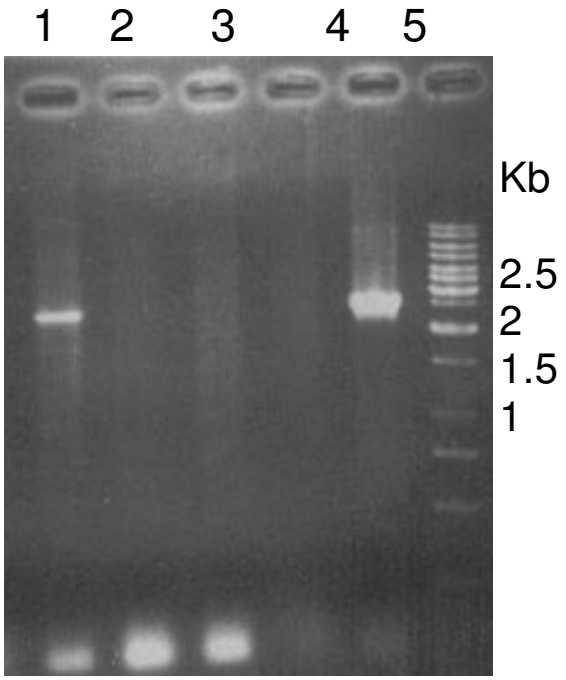

Figure 1. PCR amplification of full length PGA gene from *E. coli* isolates. Lane 1 shows a positive strain.

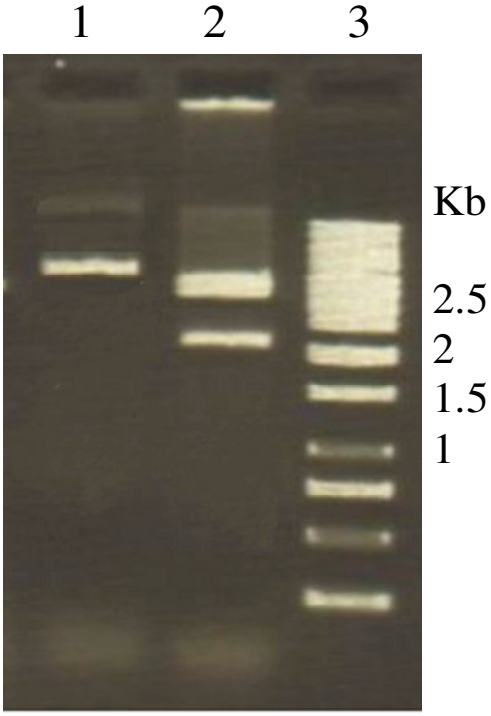

Figure 2. Cloning of the full length PGA gene from an *E. coli* isolate in pGEM-T vector. Lane 1, pGEM –PGA undigested; lane 2, the same clone digested with MscI-Xho I; lane 3, 1 Kb size marker.

were resuspended in 0.1 M phosphate buffer, pH 7.5 and incubated for half of an hour with 5 mg of penicillin G. The reaction was stopped by the addition of 3 mL of a (1: 1) mixture of 0.05 M NaOH – 20% acetic acid and centrifuged. Production of 6APA was estimated by incubation of supernatant with pDAB. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 □mol 6-APA per minute at 37°C.

RESULTS

PCR screening for PGA producing *E. coli* isolates

In this study, screening of 280 specimens (environmental and clinical specimens) for *E. coli* resulted in isolation of 180 *E. coli* bacteria. PCR screening of *E. coli* isolates for PGA production resulted in identification of 5 positive strains.

Amplification, cloning and sequencing of PGA

PCR amplification of *E. coli* genomic DNA with primers designed on conserved region of PGA gene resulted in a PCR band of 815 bp. Sequencing of the PCR products confirmed their identity as a part of PGA gene. The nucleotide sequence was deposited in NCBI Gene Bank under accession number HM011571. Amplification of full length PGA produced a PCR product of about 2500 bp (Figure 1). The PCR product was ligated to the pGEM-Teasy vector and cloning was verified by PCR and restriction digestion (Figure 2). Sequencing of cloned gene revealed that the gene consisted of 2538 bp encoding a protein of 846 amino acids. Multiple alignment of predicted amino acid sequence with PGA sequences in data base revealed a homology ranging from 90 - 94%. This shows that PGA sequence is highly conserved among *E. coli* strains.

Recombinant expression of PGA in *E. coli*

The insert containing the full length PGA was subcloned into the pET 22 b expression vector (Novagen) in frame with a C-terminal His-tag fusion for affinity purification (Figure 3). Induction with IPTG of *E. coli* BL21 transformed with expression cassette resulted in a high expression of recombinant protein as appeared in SDS-PAGE analysis of lysate of induced bacteria. Figure 4 shows Commassie blue-stained SDS-PAGE gels of *E. coli* culture before and after induction along with the bacteria without expression construct.

Biological assay of recombinant PGA expression

To compare the PGA activity of recombinant *E. coli* BL21 carrying the PGA gene on pET 22 b expression vector

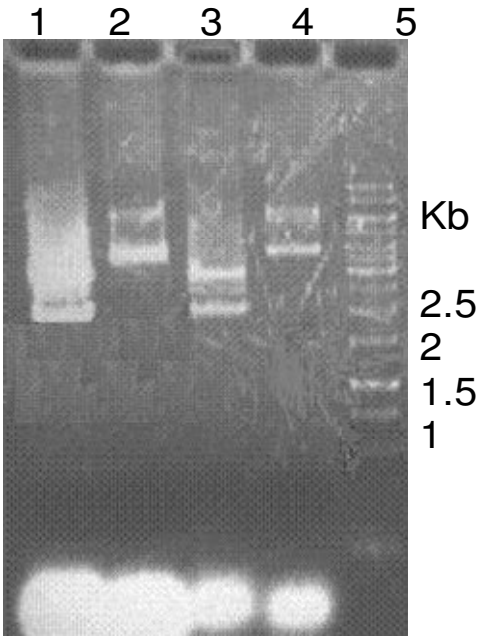

Figure 3. Subcloning of PGA gene in pET 22 b expression vector. Lane 1 and 3; undigest, lane 2 and 4; the same clones digested with Msc I and Xho I.

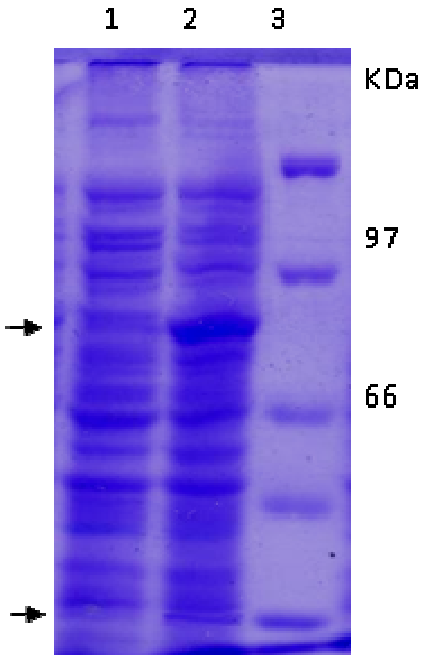

Figure 4. Recombinant expression of PGA in *E. coli* BL21. Lane 1, before induction; lane 2, after induction.

with wild *E. coli* isolate, the bacteria were cultured in media I and induced by addition of different concentration of IPTG. Culture was continued for high density (Lee, 1996), harvested, incubated with penicillin G and assayed for 6APA production. The results showed that induction with 1 mM IPTG can result in a high level of PGA activity by *E. coli* BL21 that were equivalent to 150 unit per gram (wet weight) of recombinant bacteria. This activity was about 300% more than that exhibited by wild type *E. coli*.

DISCUSSION

Biological screening methods have been used for isolation and identification of bacteria producing PGA but this method is labor and time consuming. In this project, the PCR technique was utilized for identification of penicillin G acylase producing *E. coli* strains from clinical and environmental samples. We found 5 PGA producing *E. coli* strains among 280 screened isolated. The results of our study showed that this procedure is a simple, rapid and efficient method for identification of PGA producing bacterial strains. This result was in line with previous report on application of PCR technique for identification of 7ACA positive bacterial strains (Luo et al., 2005).

The gene from one positive isolates was cloned in the 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 contains 98% homology to previously reported PGA gene from *E. coli* strains. Homology with other reported PGA genes were 96% to *Achromobacter xylosoxidans* (Cai et al., 2004) and 81% homology to *Kluyvera citrophila* (Guisán et al., 1993). The results of our study showed that the PGA gene is highly conserved among *E. coli* strains. The high conservation of PGA gene in *E. coli* isolates may indicate important benefit of this enzyme for survival of these bacteria in different environmental condition. Assay for penicillin G acylase production by *E. coli* BL21 expressing recombinant PGA showed high level of enzyme activity that was 3 times more than PGA activity of wild type *E. coli* strain. These results are consistent with previous reports indicating the advantages of recombinant DNA technology for production of PGA enzyme (Olsson et al., 1985; Garcia et al., 1986; Polderman-Tijmes et al., 2002).

Conclusion

This study showed the suitability and benefit of PCR technique for rapid detection of Penicillin G acylase in wild *E. coli* isolates from different origins. The results of our study could expand the application of PCR as a cheap and rapid method for screening of isolates with pharmaceutical and biological important characteristics. The results of this project also showed that recombinant technology could be used for high level production of PGA.

REFERENCES

Forbes BA, Sahm DF, Weissfeld AS (2006). Bailey and Scott's. Diagnostic Microbiology. Mosby company. 11th ed. The Mosby Company, St. Louis, MO.

Cai G, Zhu S, Yang S, Zhao G, Jiang W (2004). Cloning, Over-expression, and Characterization of a Novel Thermostable. Penicillin G Acylase from Achromobacter xylosoxidans: Probing the Molecular Basis for Its High Thermostability. Appl. Environ. Microbiol. 70: 2764-2770.

Demain AL (2000). Small bugs, big business: the economic power of the Microbe. Biotechnol. Adv. 18: 499-514.

Garcia JL, Busea JM (1986). An improved method to clone penicillin acylase genes: Cloning and expression in Escherichia coli of penicillin G acylase from *Kluyvera citrophila*. J. Biotechnol. 3: 187-195.

Guisán JM, Alvaro G, Fernandez-Lafuente R, Rosell CM, Garcia JL, Tagliani A (1993). Stabilization of heterodimeric enzyme by multipoint covalent immobilization: Penicillin G acylase from *Kluyvera citrophila*. Biotechnol. Bioeng. 42: 455-64.

Lee SY (1996). High cell-density culture of *Escherichia coli*. Trends Biotechnol. 14: 98-105.

Luo H, Yu H, Li Q, Shen Z (2005). Rapid Cloning and Expression of Glutaryl-7-Aminocephalosporanic Acid Acylase Genes from Soil Samples. Tsinghua Sci. Technol. 10: 529-534.

Montazam H, Farajnia S, Aboshof F (2009). Molecular cloning and Sequencing of Penicillin G Acylase from *Shigella boudii*. Afr. J. Biotechnol. 8: 1211-1213.

Ochman H, Gerber AS, Hartl DL (1988). Genetic applications of an inverse polymerase chain reaction. Genetics, 120: 621-623.

Ohashi H, Katsuta Y, Nagashima M, Kamei T, Yano M (1989). Expression of the Arthrobacter viscosus Penicillin G Acylase Gene in *Escherichia coli* and *Bacillus subtilis*. Appl. Environ. Microbiol. 55: 1351-1356.

Olsson A, Hagstrom T, Nilsson B, Uhlen M, Gatenbeck S (1985). Molecular cloning of *Bacillus sphaericus* penicillin V amidase gene and its expression in *Escherichia coli* and *Bacillus subtilis*. Appl. Environ. Microbiol. 49: 1084-1089.

Parmar A, Kumar H, Marwaha SS, Kennedy JF (2000). Advances in enzymatic transformation of penicillin to 6-aminopenicillanic acid (6-APA). Biotechnol. Adv. 18: 289-301.

Polderman-Tijmes JJ, Jekel PA, Merode VA, Floris TAG, van der Laan JM, Sonke T, Janssen DB (2002). Cloning, Sequence Analysis, and Expression in *Escherichia coli* of the Gene Encoding an α-Amino Acid Ester Hydrolase from *Acetobacter turbidans*. Appl. Environ. Microbiol. 68: 211-218.

Shewale JG, Deshpande BS, Sudhakaran VK, Ambedkar SS (1990). Penicillin Acylases: applications and potentials. Process Biochem. Int. 25: 97-103.

Shewale JG, Kumar KK, Ambekar GR (1987). Evaluation of determination of 6-aminopenicillanic acid by p-dimethylaminobenzaldehyde. Biotechnol. Tech. 1: 69-72.

Kumar Suresh R, Prabhune AA, Pundle AV, Karthikeyan M, Suresh CG (2007). A tryptophan residue is identified in the substrate binding of penicillin G acylase from *Kluyvera citrophila*. Enzyme Microb. Technol. 40: 1389-1397.

Van Langen LM, De Vroom E, Van Rantwijk F, Sheldon RA (2001). Enzymatic coupling using a mixture of side chain donors affords a greener process for ampicillin. Green Chem. 3: 316.

Wang X, Preston JF 3rd, Romeo T (2004). The pga ABCD Locus of Escherichia coli promotes the Synthesis of a Polysaccharide Adhesin Required for Biofilm Formation. J. Bacteriol. 186: 2724-2734.

Zietkiewicz E, Rafalski A, Labuda D (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics, 20: 176-83.