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ACTION OF BETA-LACTAMASE ON *E. COLI*

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ABSTRACT

Medicinal plants are of great importance to the health of individuals and communities in general. Plant essential oils are potential source of antimicrobial of natural origin. Essential oils and extracts obtained from many plants have recently gained a great popularity and scientific interest.

INTRODUCTION

E. Coli bacteria were discovered in the humans colon in 1885 by German bacteriologist Theodor Escherich Dr. Escherich also showed that certain strains of the bacterium were responsible for infant diarrhoea and gastroenteritis, an important public health discovery. Although E. coli bacteria were initially called Bacterium coli, the name was later changed to E. Coli to honor its discover.

Tem-1β-lactamase is one of the most well-known antibiotic resistance determination around. It confers to penicillins and early cephalosporins and has shown an astonishing functional plasticity in response to the introduction of novel drugs derived from these antibiotics. Since its discovery in the 1960s, over 170 variants of TEM-1- with different amino acid sequence and often resistance phenotypes-have been isolated in hospitals and clinicsworldwide.

TEM-1 β-lactamase is the most common plasmid-encoded β-lactamase in Gram-negative bacteria and is a model class A enzyme. The active site of class A β-lactamase share several conserved residues including Ser(70), Glu(166), and Asn(170) that a hydrolytic water involved in deacylation. Unlike Ser(70) and Glu(166), the functional significance of residue

Asn(170) is not well understood even though it forms hydrogen bonds with both Glu(166) and the hydrolytic water.

Among the β-lactamase, the TEM-1allele is the most common β-lactamase protect bacteria against β-lactamase antibiotics, which include penicillin and its derivatives. TEM-1 was the first β-lactamase gene to be characterized and provide resistance only to penicillin and ampicillin, while other, newer TEM alleles confer resistance to additional β-lactamase.

TEM-1 β-lactamase can accommodate insertions of random sequence in two loops surrounding its active site without compromising its activity.

TEM-type are the most prevalent β-lactamase in enterobacteria, they hydrolyses the β-lactam bond in susceptible β-lactamase antibiotics, thus conferring resistance to penicillin and cephalosporins. TEM-3 and TEM-4 are capable of hydrolyzing cefotaxime. TEM-5 is hydrolyzing ceftazidime, TEM-6 is capable of hydrolyzing ceflazidime. TEM-8/CAZ-2, TEM-16/CAZ-7 and TEM -24/CAZ-6 are markedly active against Ceftazidime.

Extended –spectrum β-lactamase (ESBL) are enzyme that confer resistance to most β-lactama antibiotics, including penicillin cephalosporins, and the monobactamaztreonam, infection with ESBL-producing organisms have been associated with poor outcomes.

Community and hospital-acquired ESBL –producing organisms in clinical laboratories can be challenging, so their prevalence is likely underestimated. Carbapenems are the best antimicrobial agent for infection caused by such organisms.

EXPERIMENTAL

Material Required

Luria-Bertania Broth and Luria-Bertani agar, Genome of E.coli was obtained from ATCC(American Type of Culture Ciliction).

Strains and Plasmid

The following strains and plasmids were used E.coli/BI 21 and E.coliDH 5α.

The above bacterial strain was routinely maintained in the laboratory, DH5-and BL21 (----) strains were maintained on Luria agar medium or in glycerol stocks for long term usage. The recombinants strains were maintained as glycerol stocks.

Enzymes and reagents

Restriction enzymes, polymerases and ligases were purchased from Ferments PCR master mix was purchased from **Fermentas.**

Solutions and buffer used

Acrylamide N,N-methylene, bis-acrylamide, sodium chloride, sodium hydroxide, glycine, sodium dodecyl sulphate(SDS), agarose, coomassie brilliant blue were purchased from SIGMA(USA) ammonium sulphate and TrisHel were purchased from SIGMA (USA).

METHODS

Cloning

Primer designing

DNA amplification requires primers, which are short oligonucleotides (around 20bp long) that several to a single standard DNA template in a unique location, thereby functioning as a start site for chain extension by DNA polymerase. Efficacy and sensitive of PCR largely depend on the efficiency of primers. The other factor being quantity of the DNA template, the mixture of chemical reagents and time-temperature program scheme.

Some criteria for primer design

Melting temperature (Tm)

It is the most crucial criterion, which determines the stability of the duplex formed between the primer and the DNA template and also the specificity of the reaction.

GC content

The GC content should be selected for stable specific binding, yet allow efficient melting.

Primer length

The length of a primer is a trade off between being sufficient for uniquencess, providing stability, and being as short as possible to minimize cost A 20-mer is considered to be optimum, but the some range around this optimum is tolerated. In addition, it has also been found that having a G or C at 3℃ temperature of the extension (this is known as GC clamp). It is also important that the primer not be able to fold onto itself and form a hairpin structure, thus inhibiting its ability to bind to the template. To avoid this, primers tested for self-complementarily, especially at the 3’ end. Finally, primers should bind to unique location within the sequence template, therefore it is reasonable to avoid common repetitive elements

or common dinucleotide and trinucleotide repeat. Following are the criteria for the selection of primer length.

1. The sequence of TEM-1 was retrieved from the database and the disordered regions were predicted.
2. Both specific forward and reverse primers were designed to amplify the full sequence.
3. The forward and reverse primers contain ndel sites respectively.

PCR Reaction

Introduction

The polymerase chain reaction (PCR) is a technique widely used to amplify a piece of DNA by in vitro enzymatic replication. Genomic DNA or any DNA material is used as template to which complementary primers bind and the region in between the primers is amplified by 5'-3' polymerase activity. As PCR Progresses, the DNA thus generated is itself used as template for replication. Most PCR methods typically amplify DNA fragments of up to 10 kb.

Chemicals

Taq Buffer, dNTPs mix, Primers, Taq Polymerase, Template DNA, Millipore Water.

Procedure

Singly colony of E.coli Strain was boiled in 10 µlitre sterile water and centrifuged. The supernatant was used for PCR.

1. Standard Reaction mixture (50 µlitre) was prepared as under.

Table 1: PCR reaction mixture set up.

Dntps (10Mm)	1 µlitre
Forward primer(25 micron M)	1 µlitre
Reverse primer (25 micron M)	1 µlitre
Taq DNA polymerase (5U/micron Litre)	0.25 µlitre
Genomic DNA (15ng/micron litre)	2 µlitre
Sterile water to make up the vol to	50 µlitre

One PCR reaction mixture was prepared having gene specific primers and genomic DNA. Lid temperature 104℃. The PCR program for amplification include the following steps:

(a) Initial Denaturation 94℃ for 5 minutes

(b) Denaturation 94℃ for 1 minutes

(c) Annealing 58℃ for 1 minutes

(d) Extension 2℃ for 1 minutes

(e) Go to step 2 for 30 cycles

(f) Final Extension 72℃ for 10 minutes

(g) Hold Extension 4℃ for 1 hour

(h) End.

Analysis of PCR Product

The amplified PCR products were resolved on Agarose gel electrophoresis.

Introduction

Agarose gel electrophoresis is a method used in molecular biology to separate DNA, or RNA molecules by size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis). Shorter molecules moves faster and migrates than longer ones. But conformation of the DNA molecule and the concentration of the gel is also a factor. The most common dye used to make DNA or RNA bands visible for agarose gel electrophoresis is ethidium bromide, usually abbreviated as EtBr. It fluoresces under UV light when intercalated into DNA. Loading buffer are added with the DNA in order to visualize it and sediment it in the gel well. Negative charged indicator like Bromophenol blue, keep track of the position of the DNA. They run at about 5000 bp and 300 bp respectively, but the precise position varies with percentage of the gel.

Chemicals

Agarose, TAF buffer, Ethidium bromide, Bromophenol blue, Glycerol or Sucrose, Standard DNA ladder.

Procedure

0.8 g of agarose was added to 100 ml 1X TAE Buffer. The mixture was heated in a microwave oven till the agarose was completely dissolved. 1micron litre of Ethidium bromide (100 mg/ml stock) was added after the solution became lukewarm. This solution was poured into the gel casting tray fitted with appropriate comb and allowed to solidify. This gel was used for the submerged electrophoresis for separating the DNA samples using 1X TAE as the running buffer at 80 V.

Gel elution of PCR product

Introduction

The DNA was resolved on agarose gel and the insert was excised with a sterile scalpel blade. It was then eluted from the gel using gel elution kit(? Germany) according to given protocol.

Chemicals

Gel Solublilization buffer, Wash buffer, Elution buffer.

Procedure

The DNA containing agarose gel silica was cut out and weighed in a micro centrifuge tube. Care was taken to cut only the agarose region that contain DNA as excess agarose gel reduces the efficiency of DNA extraction. Three volume of gel solubilisation buffer were added to one volume of agarose gel. Incubated the reaction mixture at 50℃ for 5 minutes. Melted agarose was transferred to spin column. Centrifuge for 1 minute at 11,00rpm. Flow through was discarded and 200 micron litre wash buffer was added to the column and centrifuged for 1 minute to ensure that no wash buffer remained in the column. The above step was repeated one again. The empty spin column was placed in a new 1.5 ml tube and 35 micron litre of nuclease free water was added to the centre of membrane. The column was allowed to stand at room temperature for 2-4 minutes and then centrifuged for 2 minutes to elute the DNA.

Ligation in pQE2 cloning vector

The gel purified PCR products was directly ligated in Pqe2 vector. The ligation mixture set up is given in table 2.

Table 2: Ligation reaction mixture setup.

Pqe2 vector	1 µlitre
PCR product	3 µlitre
10 X Ligase buffer	2 µlitre
T4 DNA ligase	1 µlitre
Water(to bring total volume to 10ml)	3 µlitre

Preparation of E coli DH5α competent cells

Introduction

Competence refers to the state of being able to take up exogenous DNA from the environment. Competence is not encoded in the cell's genes. Instead it is induced by laboratory procedures in which cells are passively made permeable to DNA, using conditions that do not normally occur in nature. The bacterial cell membrane are negatively charged and

so repel the negatively charged DNA from entering the cell. The positively charged calcium ion during the calcium chloride treatment mask the negative charge of the membrane and aid in the entry of the DNA into the cell during a transient heat shock. An excellent preparation of competent cells will give 108 colonies per microgram of plasmid.

Procedure

Single colony of E coli DH5α was inoculated in 10 ml of LB media and incubated at 37℃ in a shaker at 250 rpm. The overnight grown culture was inoculated in 10 ml LB media Culture was incubated at 37℃ with 250 rpm till OD 600-o.6-0.8 is reached 1 ml of Culture was transferred to precooled microcentrifuge tube and was harvested by centrifugation at 5000 rpm for 10 minutes at 4℃. Supernatant was discarded completely and pellet was resuspended in 1.5 ml of 0.1 M calcium chloride. After 45 minutes incubation on ice, the cells were again harvested by centrifugation at 5000 rpm for 10 minute at 4℃. The cells were then suspended in 150 µl cold 0.1 M $CaCl_2$.

Transformation

DNA ligation mixture was gently mix with competent cell and incubated on ice for 30 minutes. After incubation the cells were placed in water bath at 42℃ for 90 seconds(heat shock) and immediately transferred to ice 800 µl of LB medium was added to the cells and kept at 37℃ for one hour with shaking at 250 rpm. The cells were spreading on LB agar plate containing 100 µg/ml of Ampicillin. 100mM x-gal and 20 µl of 1M 1PTG. The plates were incubated at 37℃ for 12-16 hours. Colonics appeared next day and white selected and checked for the insert.

Plasmid isolation

Single colony was picked up with sterile toothpick and inoculated in 5 ml of fresh medium with Ampiciin and kept for overnight inoculation at 37℃. Plasmid DNA was isolated from Plamidplasmid isolation kit, according to manufacturer protocol given below.

3 ml culture was centrifuged and the bacterial pellet was resuspended in 250µl of P1 buffer by vortexing 250µl of P2 buffer was added and mixed gently till the colour of the suspension became blue. 350µl of buffer N3 was added and slurry mixed gently till the colour became white. The tubes were centrifuged at 12000rpm for 10 minutes at RT. The supernatant was loaded on to a spin column and centrifuged at 12000 rpm for one minutes at RT. The flow through was discarded and 500µl of buffer PB was added to the spin column and centrifuged

for 1 minutes at RT 750µl of buffer PE was added to the spin column and centrifuged at 12000 rpm for one minute at RT. Additional 1 minutes centrifugation was done at 12000rpm at RT to remove traces of PE buffer. 50µl of EB was added to the centre of the spin column and kept it on a fresh eppendr of tube and incubated at RT for 2 minute. The column was centrifuged at 12000 rpm for 2 minute at RT to collect the plasmid DNA.

Restriction enzyme digestion and analysis

The restriction analysis of the recombinant plasmid was done using appropriate restriction enzyme sites flanking the multiple cloning sites of the vectors. The reaction was set as follows:

Table 3: Restriction digestion reaction mixture.

Plasmid DNA	8 µl
10 X reaction buffer	2 µl
Ndel	1µl
Hindlll	1µl
Water to make volume upto	20µl

Expression of TEM-1 Protein

(a) Sodium dodecyl sulphate Polyacrylamide gel electrophoresis

Principles

Protein molecules are separated on the basis of molecular weight in a polyacrylamide Gel matrix. SDS binds to denatured protein giving it a net negative charge allowing it to migrate towards the anode in electrophoresis.

Materials

The plates were assembled according to manufacture's instructions. The volume of different gel component required to cast 14% resolving gel was mixed as follows:

Water	3.3 ml
30 % acrylamide	3.0 ml
1.5 M Tris(pH 8.8)	5 ml
10 % SDS	0.1 ml
10 % APS	0.1 ml
TEMED	0.004 ml

(Without delay, the mixture was rapidly swirled to mix)

The acrylamide solution was poured into the gap between the glass plates leaving sufficient space for the stacking gel. After polymerization was completed, the prepared stacking gel

mixture was filled up in the remaining space over the separating gel, and the comb appropriately placed. The stacking gel was allowed to solidify. The stacking gel mixture was as follows:

water	2.5 ml
30% acrylamide mix.	0.7 ml
5M Tris(Ph 6.8)	0.5 ml
10 % SDS	0.01ml
10 % APS	0.01 ml
TEMED	0.004 ml

Staining SDS- Polyacrylamide gel with Coomassic Brilliant Blue R-250

1. The staining solution was prepared by dissolving 0.25 g of Coomassic Brillian Blue R250/100 ml of methanol: acetic acid solution (5:4:1 methanol :water: glacial acetic acid)
2. The gel was immersed in the staining solution and allowed to stand at room temperature for 4 hrs.

Destaining of Gel

1. The gel was destained by soaking in methanol: acetic acid solution without the dye for approximately 6 hrs, changing destaining solution 3-4 times.
2. The gel stored after destaining in distilled water for documentation.

Purification

General purification methods used for protein purification

There are several chromatographic methods used along with some precipitation method to purify proteins from the mixture of proteins or starting. These techniques are used by different characteristics properties of proteins i.e. molecular size (Size –exclusion chromatography), net molecular charge of protein.

Affinity chromatography

Affinity chromatography is widely used for separation of protein/biological molecular based on the biological interaction between antigen and antibody, enzyme and substrate, or receptor and ligand. Affinity chromatography combines the size fractionation capability of gel permeation chromatography with the ability to design a stationary phase that reversibly binds to a known subset of molecules. Usually the starting point is an underfined heterogenous group of molecules in solution. Such as a cell lysate, growth medium or blood serum. The molecules of interest will have a well-known and defined property which can be exploited during the affinity purification process. The protein itself can be thought of as an entrapment,

with the target molecules becoming trapped on a solid or stationary phase or medium. The other molecules in solution will not become trapped as they do not possess this property. The solid medium can then be removed from these mixture, washed and the target molecule released from the entrapment in a process known as elution.

RESULT AND DISCUSSION

This gel shows the protein bands obtained on the SDS-PAGE after the induced by different 1PTG concentrations (viz.0.2 mM, 0.4 mM, 0.5mM, 0.8 mM, 1.0 mM). The best result 1PTG concentration 0.5 mM whereas at 1PTG concentration 0.2 mM and 0.4 mM the protein yield was very low and at 1PTG concentration 0.8 mM and 1.0 mM the recombinant protein form inclusion body.

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