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BIOTRANSFORMATION OF AMPICILLIN ANTIBIOTIC MOLECULE USING SOIL DERIVED BACTERIUM BELONGING TO *ALCANIVORAX* SP.

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Abstract – Ampicillin was selected as a representative beta lactam antibiotic for this investigation. After preliminary screening, an antibiotic resistant bacterium was isolated from soil. This bacterium was identified using 16S rRNA gene sequences as *Alcanivorax* sp. The beta lactamase gene responsible for the resistance was also detected in in this bacterial strain. The products of transformation were analyzed using ESITOFMS. MS analysis resulted in mass fingerprints of the metabolites formed during transformation of ampicillin by bacterial strain. Initially this bacterial strain transformed ampicillin into ampicilloic acid with the help of beta-lactamase enzyme. In the present study it was found that ampicillin resistant bacterium exhibit beta lactamase gene and in biotransformation experiment it was proved that this gene produces beta-lactamase enzyme, which opens-up beta-lactam ring of the antibiotic. This study highlights the importance of soil derived bacteria in the transformation of antibiotic molecules.

INTRODUCTION

The antibiotic resistance in environmental bacteria is a growing concern because of its link to the human health. There are some reports on antibiotic resistance of bacteria isolated from soil (Armalyte *et al*., 2019). It is well-known that this resistance could be due to anthropogenic factors in the form of antibiotics being used in clinics and in the agriculture sector. In view of this, the soil was selected to identify antibiotic resistant bacteria and to investigate further the transformation of ampicillin antibiotic.

Soil bacteria and antibiotic resistance

In the soil bacteria are the most abundant microbial forms. There can be billions of these single celled organisms' bacteria in a single gram of soil. Populations of bacteria can boom or bust in the space of a few days in response to changes in soil moisture, soil temperature or carbon substrate. There is a widespread presence of high-level antibiotic-resistant bacteria in the aforementioned soils. Soils are important reservoirs of diverse antibiotic resistance and antibiotic production genes, and antibiotic resistance genes are able to spread rapidly in clinical settings through horizontal gene transfer (Nesme and Simonet, 2015). Julija *et al.* 2019 reported Microbial Diversity and antimicrobial resistance profile in microbiota from soils of conventional and organic farming systems.

The mechanism of antibiotic resistance in bacteria is known and is attributed to the mutation and the genetic machinery present in certain bacteria to produce enzymes, which can destroy specific antibiotics. As far as the enzymes are concerned the bacterial resistance against β-Lactam class antibiotics would be the classic example. This is a broad class of antibiotics that contain a β-lactam nucleus in its molecular structure. The major antibiotics in this class are penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems. Generally these antibiotics inhibit the cell wall synthesis by the bacterial pathogens. Bacteria often develop resistance to β-lactam antibiotics by synthesizing beta-lactamase, an enzyme that attacks the β-lactam ring. β-lactamase enzymes are responsible for break down of lactam ring.

In the present investigation, the Ampicillin a representative Beta lactam antibiotic to check bacterial resistance and further biotransformation

studies. The antibiotic resistance in bacteria and presence of Beta lactamases has been well documented in previous studies. In 2002, Brinas *et al.* found that ampicillin resistant *Escherichia coli* isolate from foods, humans, and healthy animals posses Beta-lactamases. A similar observation was made by Olsen *et al.* in 2004, where they could detect beta-lactamases among ampicillin-resistant *Escherichia coli* and *Salmonella*, isolated from food animals.

MATERIALS AND METHODS

Isolation of soil bacteria

Soil sample was collected from the garden during May 2019. The soil sample was immediately transferred into sterile plastic containers and transported to the laboratory in a cold sterile condition and then stored at 4 °C. Soil sample was diluted with sterile distilled water. Up to 10-9 dilutions were made in total volume of 10 mL of DW. 100 µL from different dilutions was placed on nutrient agar plates. These plates were then incubated at 30 °C and observed for microbial growth ranging from 24 hrs to one week. From the incubated plates, bacteria isolates were selected by considering their morphology and pigmentation. 10 bacteria were purified further on nutrient agar plates.

Screening of soil bacteria for ampicillin resistance

Total 10 soil derived bacterial isolates were checked for their ampicillin resistance profile using Ampicillin AMP 10mcg discs (HiMedia laboratories, India). Mueller Hinton agar (HiMedia # M1084-500G) medium plates used for testing ampicillin resistance were overlaid with 1% nutrient agar and 100 µL of cultures for growth of bacteria. After solidification of the medium, discs were placed in plates. Plates were incubated at 28 °C and after 24 hrs the antibacterial activity of ampicillin was evaluated by measuring the zone of inhibition against the test organisms.

Phylogenetic analysis of ampicillin resistant bacteria

Amplification of 16S rRNA gene of a ampicillin resistant soil derived bacterium was carried out, following the method of Zhou *et al.*, 2009. The universal primers 27f (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1385r (5′ CGG TGT GTA CAA GGC CC 3′), corresponding to *Escherichia coli* 16S rDNA numbering were used for the amplification. PCR reaction was performed in a total volume of 50 µL containing 25 µL of PCR master mix (Sigma # P4600), 2µL of template DNA (diluted to 10ng/µL), 2µL of forward primer (10 µM), 2µL of reverse primer (10µM) and 19 µL of nuclease free water in a PCR tube. PCR was carried out at annealing temperature 52°C by using thermal cycler..

The obtained PCR product was further purified by using PCR product purification kit (Qiagen QIAquick 96 PCR purification kit, Catalogue No. 28180). PCR product was sequenced using 27F as a sequencing primer. The sequences (approximately 700 bp) were recorded for further analysis. The sequences of PCR product were analyzed by using Basic Local Alignment Search Tool (BLAST). Nucleotide-nucleotide BLAST was carried with facility of National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST).

Amplification of B-lactamase gene

Bacterium (S3) having resistance to the antibiotic ampicillin were selected for the detection of Beta-lactamase gene by following PCR based method described by Gonzalo and Quesada (2000). Bacterial DNA was isolated by using Quiagen Kit (Cat. No. 69504). These bacterial DNA samples were used as templates for the amplification of Beta-lactamase gene. The primers Amp5′ (5′-TGAAGATCAGTTGGGTG-3′) and Amp3′ (5′-CCCCGTCGTGTAGATAA-3′) were used to amplify specifically a 690 bp band (Ac. No. X65304) from the open reading frame of the *E. coli* Beta-lactamase gene (Gonzalo and Quesada 2000). These primers were synthesized by Sigma-Aldrich, India. PCR reaction mixture of 50 µL was prepared by adding 25˜l of PCR master mix, 2µL of template DNA (diluted to 10ng/µL), 2 µL of forward primer (10 µM), 2µL of reverse primer (10 µM) and 19µL of nuclease free water in a PCR tube. PCR was carried out in Takara thermal cycler with initial denaturation at 92 °C for 5 minutes, followed by 30 cycles of each 91 °C for 1minute, 45 °C for 1minute, 72°C for 2minutes. Final extension at 72 °C for 7 minutes. Sample was stored at 4 °C for further use. To check the size of PCR product, electrophoresis was carried out with 1.2% agarose gel and 1kb DNA ladder.

PCR products of the partial sequence of B-lactamase gene were purified by using AxyPrep TMPCR Cleanup kit (Axygen- AP-PCR-50) and

sequenced by using Amp5 as a sequencing primer. The obtained sequences were further analysed by using NCBI BLAST. The protein database was searched using the nucleotide sequences obtained by PCR amplification of the ampicillin resistant (AMPR) gene using NCBI blast x. The first three sequences showing maximum identity and highest E-value were selected. Amino acid sequence alignment of the partial beta-lactamase gene of 4 antibiotic resistant bacteria and beta-lactamase gene obtained from *E. coli* (NCBI Accession no. AAP93845.1) was carried out using CLUSTAL W (Felsenstein, 1985; Saitou and Neil, 1987; Tamura *et al.*, 2011).

Biotransformation of Ampicillin by using soil derived bacterium

The ampicillin resistant bacterium (S3) was used for biotransformation. For fermentationof this bacterium nutrient broth (HiMedia XXXX) was used. After inoculation, the culture fermented at 28 ^{o}C in shaking incubator for 48 hours. After 48 hours, the bacterial culture was centrifuged in cooling centrifuge at 8000rpm for 10min to obtain cell pellet. The supernatant was discarded to remove carbon source. The pellet was further used for biotransformation.

Sodium phosphate buffer of pH (7.2) was used as a base for the reaction in which 250 mg of ampicillin (ampicillin sodium salt, HiMedia Cat No. TC021-1G) was added and dissolved properly. Obtained pellet was rinsed with small amount of buffer, dissolved by vortexing and inoculated in 1000 mL of buffer. It was incubated for 6 hours at 28 ^{o}C in shaking incubator. Methanol (200 mL) was then added to stop the reaction by inhibiting the cell activity. Cells were separated by passing through 0.22 µm filter paper (Millipore GSWP04700) and the filtrate was concentrated using Rotary evaporator. A saturated solution of the concentrated sample was made and passed through XAD-2 resin. The resin was washed with distilled water and further eluted with Methanol. Methanol washings were collected and concentrated to obtain crude product.

Analysis of transformed products

The Mass spectra of the transformed products were obtained using electrospray ionization-time of flight mass spectrometry (ESITOFMS). The crude product was dissolved in methanol to prepare dilute solution (approx. 1µg/ml). This solution was directly injected on to ESITOFMS at potential of 5ev.

RESULTS

Ampicillin resistant bacteria

The antibacterial assay of ampicillin was performed by using 10 soil derived bacterial cultures (S1 –S10). Out of these cultures, one bacterium was found to be resistant to ampicillin at 10mcg/disc concentration. This ampicillin resistant bacterium was S3. This ampicillin resistant bacterium was further used for biotransformation of Ampicillin antibiotic.

Phylogenetic analysis

Ampicillin resistant bacterium was identified by using 16S rRNA gene sequences. The bacterium S3 obtained from the soil showed 100% sequence similarity with *Alcanivorax* sp. (NCBI accession No. JF304812.1) (Table 1).

Table 1. Phylogenetic analysis of antibiotic resistant soil derived bacterium.

Isolate	Source	Nearest Phylogenetic neighbour	% Similarity
S3	*Haliclona* sp.	*Alcanivorax* sp.	100

Detection of Beta-lactamase gene

The antibiotic resistant bacterium S3 was found to have µ-lactamase gene. The primers, which were used to amplify this specific gene confirmed the presence of this gene in S3 bacterial culture. These results are given in Figure 1.

The gene sequences showed maximum similarity with beta-lactamase [*Citrobacter freundii*], beta-lactamase [*Klebsiella pneumoniae*] and beta-lactamase [*Escherichia coli*]. Interestingly beta-lactamase gene of all four antibiotic resistant bacteria showed 100% homology with the *E.coli* beta-lactamase gene. The results are shown in Table 2.

Mass spectroscopic analysis

The transformation of ampicillin was analyzed using ESITOFMS. ESITOFMS analysis resulted in mass fingerprints of the metabolites formed during transformation of ampicillin by S3 bacterial strain and are represented in Fig 3. Several ion peaks due to degradation products were observed. The four mass spectra (Fig. 4) had significant differences between them indicating different mechanism for metabolism of ampicillin by each strain. A

Table 2. BLAST analysis of Beta-lactamase gene sequences. The sequences showed maximum similarity with beta-lactamase [*Citrobacter freundii*], beta-lactamase [*Klebsiella pneumoniae*] and beta-lactamase [*Escherichia coli*].

Bacterial code	NCBI Accession No.	E value	Max identity	Description
NIO 00084	AAP93842.1	2e-135	100%	beta-lactamase [*Citrobacter freundii*]
	AAP93840.1	2e-135	100%	beta-lactamase [Klebsiella pneumoniae]
	AAP93845.1	2e-135	100%	beta-lactamase [*Escherichia coli*]

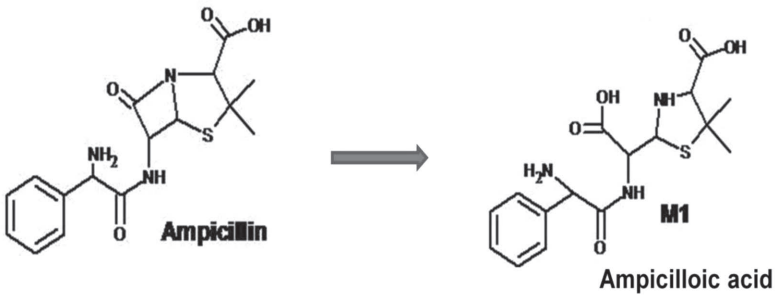

Fig. 1. Biotransformation of Ampicillin antibiotic using soil derived bacterium S3. Here S3 is converting Ampicillin to Ampicilloic acid.

pseudomolecular ion [M+H]+ at m/z 368 in spectra 4a, 4c and 4d was suggestive of ampicilloic acid (benzyl penicilloic acid of ampicillin), a hydrolytic product formed by opening of the μ-lactum ring, which is a basic function of μ-lactamase enzyme.

DISCUSSION

The soil derived bacteria are explored for various biotechnological applications. The biology and physiology of bacteria from this source has elicited considerable interest among researchers investigating soil derived bacteria for bioprospecting. In recent years, soil has been considered as a rich reserve of microorganisms, having potential in drug discovery program. Though there are several reports on bioactive potential of soil-derived bacteria, little is known about their antibiotic resistance related to the antibiotic resistance gene. Several reports show that the soil could be served one of the environmental reservoirs of the antibiotic-resistance bacteria (Armalyte *et al*., 2019). After considering all the previous reports related to this concept, the present investigation was planned to study antibiotic resistance in soil derived bacteria and further investigated the gene responsible for the same. The tramsformation of antibiotic using soil derived bacterium bacteria was also studied to understand the mechanism of antibiotic degradation.

In the present investigation, antibiotic resistant bacterium was detected from the soil belonging to the genus *Alcanivorax* sp. Ampicillin, a broad spectrum beta-lactam antibiotic having applications in the treatment of respiratory, gastrointestinal, urogenital tract and other infections was selected for antibiotic resistance as well as further transformation studies. Interestingly, out of 10 bacterial isolates obtained from the soil, one bacterium (S3) showed resistance to the antibiotic ampicillin. This bacterium showed 99–100% sequence similarity with *Alcanivorax* sp. These results highlight that soil is a natural reservoirs of antibiotic resistant bacteria. Most of the antibiotic resistant genes have been found to be linked with clinical and agricultural use of antibiotics. It is proved that the antibiotics used in clinics contribute towards antibiotic resistance in environmental bacteria. Unfortunately very little is known about the environmental reservoirs of antibiotics resistant bacteria. Aminov 2009 nicely described the Role of Antibiotics and Antibiotic Resistance in Nature.

The selected antibiotic compound in this investigation has beta lactam ring. There are some reports on lactum compounds, isolated from soil derived microbesIn order to understand mechanism of antibiotic degradation and metabolic products, the antibiotic resistant soil derived bacterium was used for biotransformation studies of ampicllin. For detailed understanding of transformation mechanism, we describe a metabolomics based approach for the detection of several metabolites of selected bacterial strains derived from the soil, using ESITOFMS data. Ampicillin has molecular weight of

349 amu and certain molecular ions in the region 300-450 amu were assigned with the plausible structures by making minor changes in the structure of ampicillin. From the mass spectrometric studies it is revealed that the S3 bacterial strain initially transform ampicillin into ampicilloic acid, thus confirming the presence of beta lactamase enzyme as also detected from PCR studies.

This investigation provides important preliminary information about antibiotic resistance in bacteria in soil. These antibiotic resistant bacteria exhibit genetic machinery to produce enzymes(s) for the transformation of antibiotics. It is reported that the antibiotic resistance gens have been evolved through ecological interactions as well (Aminov *et al.,* 2007).

In the present study it was found that ampicillin resistant bacterium exhibit beta lactamase gene. Beta-lactamases gene has also been detected previously in ampicillin-resistant *Escherichia coli* isolates from foods, humans, and healthy animals (Brinas *et al.,* 2002). Beta-lactamase enzyme originated more than two billions year ago, with some serine B-lactamase being present on plasmids for millions of years, well before the modern use of antibiotics (Haeggman *et al.,* 2004; Hall and Barlow 2004; Garau *et al.,* 2005; Aminov *et al.,* 2009). Majiduddin *et al.* has reported the molecular analysis of beta-lactamase structure and function.

In the biotransformation experiment it was proved that this gene produces beta-lactamase enzyme, which opens-up beta-lactam ring of the antibiotic. In the transformation experiment, first transformed product was ampicilloic acid. This investigation highlights the importance of soil derived bacteria for the transformation of antibiotic molecules.

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