Chapter 5:

Revealing the role of New Delhi Metallo-β-lactamase in metabolism of β-lactam antibiotics as growth substrate

5.1 Introduction

Antibiotics have always been reckoned as one of the wonder drug discoveries of the last century. This is true but increasing antibiotics-resistance in pathogenic bacteria with the associated decrease in the development of new antibiotics drugs threatens us to the return to the dark ages of the pre-antibiotics era. The protective mechanisms in bacteria that have developed include preventing entry of the antibiotics, producing hydrolytic enzymes that destroy or modify the antibiotic drugs, or making changes to the antimicrobial target sites. Therefore, the antibiotic-resistance mechanism of bacteria also could be considered to simply symbolize the Darwinian evolutionary theory. Bacteria naturally developed a resistance mechanism against the microorganism-derived antimicrobial molecules for survival and make many copies of itself in a successive generation. A wide range of biochemical and physiological mechanisms has developed in bacteria to make resistance against antibacterial compounds. In the specific case of antibacterial agent, the complexity of the processes that help to emergence and distribution of resistance can't be exaggerated, and the lack of basic knowledge on resistance mechanism is one of the primary reasons that there has been so little significant success in the effective prevention and control of resistance development.

Now microorganisms are not only developing resistance mechanisms against the antibiotics molecules but it also developed a new way they eat antibiotics as food. Despite the lack of appropriate information on antibiotics-use, 100-200 thousand tons of antibiotics used worldwide every year (Wise, 2002; Kummerer, 2003) and now also recognized have an additional role of antimicrobials. These new additional roles have been developed by bacteria and through these new roles, antibiotics offer carbon and nitrogen or only carbon sources of bacteria. However, remarkable only few works has been reported on the biochemistry of antibiotic catabolism. Recently a research work on penicillin catabolism was published and they find the conserved pathway for penicillin catabolism involving initial hydrolysis catalyzed by β-lactamases the same enzymes that are widely responsible for resistance to the penicillins and β-lactam group of antibiotics (Crofts *et*. *al*., 2018). Based on antibiotics origin antibacterial compounds are classified into three types, natural, semi-synthetic and synthetic. The present period semi-synthetic and synthetic antibiotics are more prescribed drugs. Semi synthetics antibiotics drugs like "Ampicillin" was

introduced for mankind more than 60 years ago. When the amino group added into the benzylpenicillin molecule a new molecule was developed called ampicillin (Witting and Smith 2011). Ampicillin was placed in β-lactams group, which include successive generations of β-lactams (including cephalosporin, and carbapenems) remain the most important Class of antimicrobial agents. Penicillin is a natural compound and these produced by microorganisms long before human use, and hence antibiotic-catabolism may well have ancient origins. Based on the previous study, penicillin catabolism in bacteria proceeds via initial β-lactamase-mediated hydrolysis to originate benzylpenicilloic acid, which acts as a substrate for amidase and benzylpenicilloic acid converted into phenylacetic acid. At the end of the process phenylacetic acid can then undergo conversion into acetyl coenzyme A (CoA) and succinyl CoA via an established process of phenylacetic acid catabolism (Crofts *et. al*., 2018; Teufel *et. al*., 2010). On the other hand, ampicillin is semi-synthetic compounds and it does not easily catabolized due to the amino group, but when this amino group shall be removed then it will easily be catabolized by bacteria. Serine β-lactamase enzyme can only hydrolyze ampicillin molecules and then they only converted into an inactive form, these hydrolyzed product can act as substrate molecules for amidase enzymes, but not make a product for PAA pathway. Because after amidase hydrolysis originates a benzylpeniclloic acids with an amino group do not convert into phenylacetic acid (Figure 5.1). In brief, we can say that for β-lactam antibiotic catabolism required three components and these components are (i) β-lactams resistance genes (ii) amidase genes and (iii) phenylacetic acid catabolism enzymes encoding genes. When these three components shall be present in any microorganism, then antibiotics molecules will be catabolized by those microorganisms. Previous reports have explained only natural antibiotics (penicillin) catabolism, but in this study we explain ampicillin catabolism in MBL positive strain which was isolated from Mahananda and Karala River of Siliguri and Jalpaiguri of West Bengal. All MBL positive isolates showing unexpected high MIC value against penicillin as well as ampicillin molecules. Simply this result indicates MBL positive strain used β-lactam antibiotics as a food (carbon and nitrogen molecules) for growth. Based on NDM-1 and ampicillin binding study, hypothetically we recognized an additional role of NDM-1 in ampicillin catabolism; they could be provided with a carbon and nitrogen source to bacterial cells.

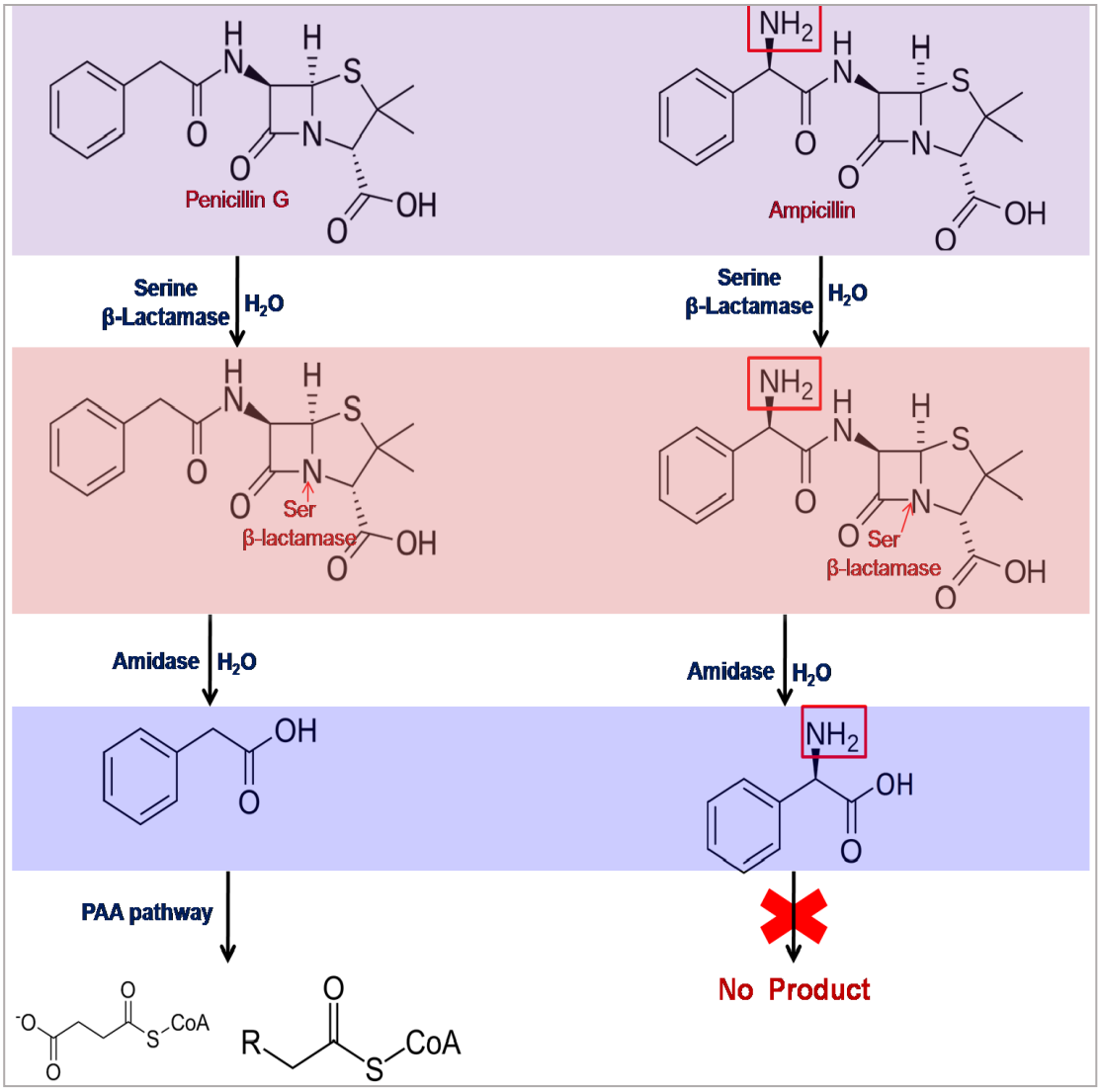

Figure 5.1: Difference between penicillin and ampicillin molecule and their catabolic pathways

This chapter is the soul of the whole study and the first and most important aim of this chapter was to establish a new role of New Delhi Metallo-β-lactamase in the catabolism of ampicillin antibiotics as a growth substrate. We demonstrate that strategy by recombinant *E. coli* DH5α strain, enabling it to grow using ampicillin as its sole carbon source. The second aim of this chapter was to demonstrate the utilization of ampicillin as a sole carbon source in MBL positive strain. All the MBL positive strains were used because all the bacterial strain carries New Delhi Metallo-β-lactamase genes. The third aim of this study was to revelation of ampicillin catabolic pathway in the draft genome of MR 02

strain, although these MR 02 strain was also used ampicillin as a sole carbon source for growth and the whole genome is available, so, this strain can be used as a model strain for analysis of β-lactams catabolic responsible genes and their expression (RNA-seq analysis).

5.2 Materials and Methods

5.2.1 Growth studies of MR 02 demonstrate utilization of ampicillin as the sole source of carbon, nitrogen, and energy

To study the utilization of ampicillin as carbon and nitrogen sources, the inoculum was prepared as follows. MR 02 was inoculated in 10ml Luria Broth containing 50µg/ml Imipenem and was incubated at 30°C in shaking incubator for 18 hrs. 18 hrs grown MR 02 culture was centrifuged in 1.5ml tube at 3000 rpm for 5 minutes and the supernatant was discarded. Then 1.5 ml fresh autoclaved distilled water was added, the cell pellet was dissolved in it following centrifugation at 3000 rpm for 5 minutes and again the supernatant was discarded. The same process was repeated for the second time. The culture was washed a total of two times in minimal media lacking a carbon and nitrogen sources. After that, the fully washed cell pellet was dissolved in 1.5 ml fresh minimal media. From this stock, 1% (200µl) inoculum was taken to inoculate each 100 ml Erlenmeyer flasks containing 20 ml minimal media with different experimental compositions.

The composition of the minimal media used in this study was as follows:

(I) Ampicillin as a sole source of carbon and nitrogen

(II) Ampicillin was used only as a sole source of carbon

For growth on ampicillin as both carbon and nitrogen sources, no dextrose and no NH_4Cl were added in minimal media, but the only different concentration of ampicillin (Himedia) 2 g/L, 4 g/L, 6 g/L with EDTA and without EDTA was added. For growth on ampicillin as the only carbon source, no dextrose but only different concentration ampicillin 2g/L, 4g/L, 6g/L, with EDTA and without EDTA was added. Minimal media were prepared consisting of (per litre) 3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 5 g/L NaCl, 5 g/L NH_4Cl, and 100 mg/l $MgSO_4$. The pH value was adjusted to 7.0 ± 0.2 and sterilized by autoclaving. After inoculation flasks were incubated at 30°C for 216 hrs in a shaking incubator at 120 rpm. Growth was monitored after every 24 hrs at 600 nm in a spectrophotometer (BMG SPECTROstar nano).

The growth of MR 02 in the minimal media containing NH_4Cl with and without EDTA was studied to test the effect of EDTA. All growth experiments included triplicate independent cultures, and OD_{600} values were evaluated as averages with standard error. Growth data were plotted using Microsoft Excel.

5.2.2 Identification of β-lactam catabolism-responsive genes from the draft genome of *Pseudomonas* sp. MR 02

Open reading frames (ORFs) of β-lactam catabolic genes (resistance-associated, catabolism associated and phenylacetic acid catabolism) were identified from the draft genome of MR 02. Sequence lengths were again analyzed by additional BLASTP via the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST).

5.2.3 Establish a new role of *bla*$_{NDM-1}$ in the β-lactam catabolism pathway

Our hypothesis is that if a bacterium will have NDM-1 gene with amidase(s) and the pathway to catabolize phenylacetic acid (PAA), then it will use ampicillin as a carbon and energy source. The amidase(s) and pathway to catabolize phenylacetic acid (PAA) are naturally present in the *E. coli* cell (Teufel *et. al*., 2010; Priyadarshini *et. al*., 2007) and when we will clone and express the NDM-1 gene in the *E. coli* cell, then recombinant cell will also use ampicillin as the carbon source. Thus, the speculation was validated with experiment.

5.2.3.1 Amplification of *bla*$_{NDM-1}$ gene *Pseudomonas* sp. MR 02

The *Pseudomonas* sp. MR 02 *bla*$_{NDM-1}$ full length (813bp) gene was amplified by Polymerase Chain Reaction using primers *Eco*RI (5′-GGGAATTCATGGAATTGCCCAATATTATG-3′) and *Pst*I (5′-AACTGCAGTCAGCGCAGCTTGTCGGCCAT-3′) (Tada *et. al*., 2014). The forward and reverse primers were flanked by the specific restriction endonuclease sites used for cloning the amplified fragments. The reaction mixture and thermal cycle profile for PCR amplification described in previous chapter 2. The Amplified product was checked in 1.5% agarose gel by gel electrophoresis.

5.2.3.2 Cloning vector pHSG398

The plasmid vector pHSG398 (Takara, Japan) of 2227bp length, was used for the cloning of full-length PCR amplified *bla*NDM-1 gene product. pHSG398 is a pUC-type

bacterial cloning vector with a chloramphenicol resistance marker gene. This vector also contains multiple cloning sites (MCS) with the *lacZ* genes (Figure. 5.2).

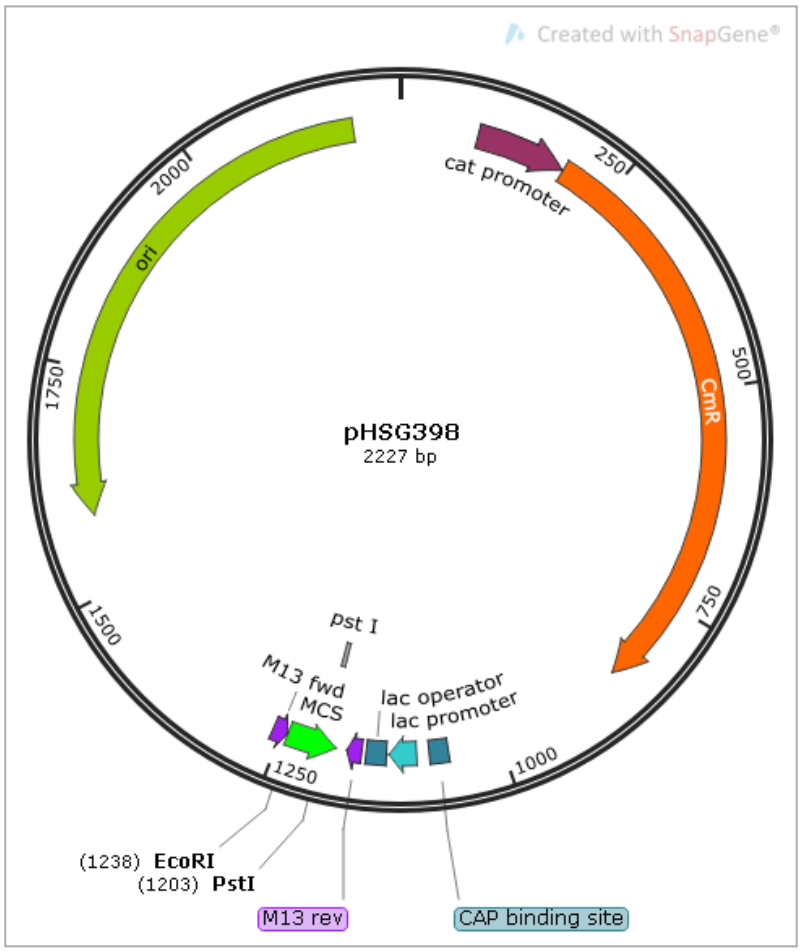

Figure 5.2: A schematic representation of plasmid vector pHSG398 (2227bp)

(Vector map were created in SnapGene tool)

5.2.3.3 Restriction endonuclease (RE) digestion of PCR amplified *bla*$_{NDM-1}$ genes and pHSG398 Plasmid

Restriction double digestion of the amplified PCR product and plasmid vector was carried out using the two endonuclease restriction enzymes *Eco*RI and *Pst*I. The reaction mixture for double digestion was prepared separately for both DNA samples as per the manufacturer protocol (Thermo Fisher Scientific). For this 2.5µl (15 units) *Eco*RI and 5µl (30 units) *Pst*I of required restriction enzymes was added to 1µg DNA samples with tango buffer supplied by the manufacturer and the mixture was incubated overnight at 37°C. The

digested DNA samples were run on the 1% agarose gel to confirm the success of the restriction digestion reaction.

5.2.3.4 Gel extraction of RE digested DNA products

The double digested PCR DNA and vector DNA bands were cut out from the agarose gel (low melting agarose) with a sterile scalpel. The DNA bands from the gel were extracted and purified by the G Sure DNA purification kit (GCC Biotech) follow manufacture instruction. Finally, the purified DNA products were eluted in the nuclease-free water separately and thereafter, the purified DNA products were stored at -20°C for further cloning work.

5.2.3.5 Preparation of competent cell

The competent cells were prepared for the transformation of the recombinant vector (pHSG398+NDM-1) is followed Mandel and Higa classic experiment method (Mandel & Higa, 1970) Chemical use in this method were described in previous chapter 2.

5.2.3.6 DNA ligation

The gel-purified double digested PCR products were ligated with double digested gel-purified pHSG398 vector in molar ratio 1:3 in an appropriate buffer and T4 ligase enzyme (GENEI). The mixture was incubated at 4°C overnight. Thereafter, the ligated DNA products were transformed into *E. coli* DH5α competent cells.

5.2.3.7 Transformation

The chemically prepared competent cells were transformed with the ligated recombinant vector (pHSG398+ bla_{NDM-1}) product via the heat shock method. 20µl (100ng DNA) ligation mixture was added aseptically to the 100 µl competent cells and mixed by gentle tapping and following the transformation method described in previous chapter 2. The transformants were spread onto the LB agar plate containing imipenem (10 mg/l) and chloramphenicol (50 mg/l). The plate was incubated at 37°C for overnight.

5.2.3.8 Screening of the recombinant by PCR methods

Confirmation of the cloned bacterial isolates was done by amplification of the conserved region of bla_{NDM-1} genes by PCR methods followed by PCR method described in previous chapter 1.

5.2.3.9 Determination of minimum inhibitory concentration (MIC) of recombinant *E. coli* DH5α strain

MIC values of the recombinant *E. coli* DH5α (pHSG398+bla_{NDM-1}) were determined by the broth macrodilution method as per CLSI guidelines (Wayne 2017). The experiment was set up in triplicates in the test tube with 3ml MH broth media containing different dilution of antibiotics and 10^6 recombinant *E. coli* DH5α (pHSG398+ bla_{NDM-1}), and wild type *E. coli* DH5α cells were used as a control. The tubes were incubated at 37°C for 16-18 hrs. Thereafter bacterial growth was determined by measuring at 600 nm via spectrophotometer (BMG SPECTROstar nano).

5.2.2.10 Catabolism of ampicillin by recombinant *E. coli* DH5α cell

To study the utilization of ampicillin as carbon sources by recombinant strain of *E. coli* DH5α (pHSG398+bla_{NDM-1}) inoculums and minimal media were prepared as the following method described above section 5.2.1. Ampicillin was used as the carbon source and the concentrations of ampicillin with minimal media used were 0.1 g/L. *E. coli* DH5α+ pHSG398 and *E. coli* DH5α+pGEM-T strain used as negative and positive control respectively. After inoculation flasks were incubated at 37°C for 48 hrs in a shaking incubator at 120 rpm. Bacterial growth was measured by CFU counting method. Samples were taken for plating on LB plates at 12 hrs interval to 48 hrs after starting the experiment. All growth experiments included triplicate independent cultures, and CFU values were evaluated as averages with standard error. Growth data were plotted using Microsoft Excel.

5.2.4 Transcriptome sequencing and bioinformatics analyses of *Pseudomonas* sp. MR 02 to determine expression of gene responsible for ampicillin-catabolism

5.2.4.1 Preparation of sample for transcriptome sequencing

For transcriptome RNA sequencing (RNA-Seq), the sample *Pseudomonas* sp. MR 02 was grown under two different experimental conditions, one with glucose (6 g/L) (control) and another one with ampicillin (6 g/L) (test/experimental) as carbon source containing minimal media (described in section 5.2.1). Culture flasks were incubated at 30°C with aeration until cultures attained log phase and turned turbid (360 hrs).

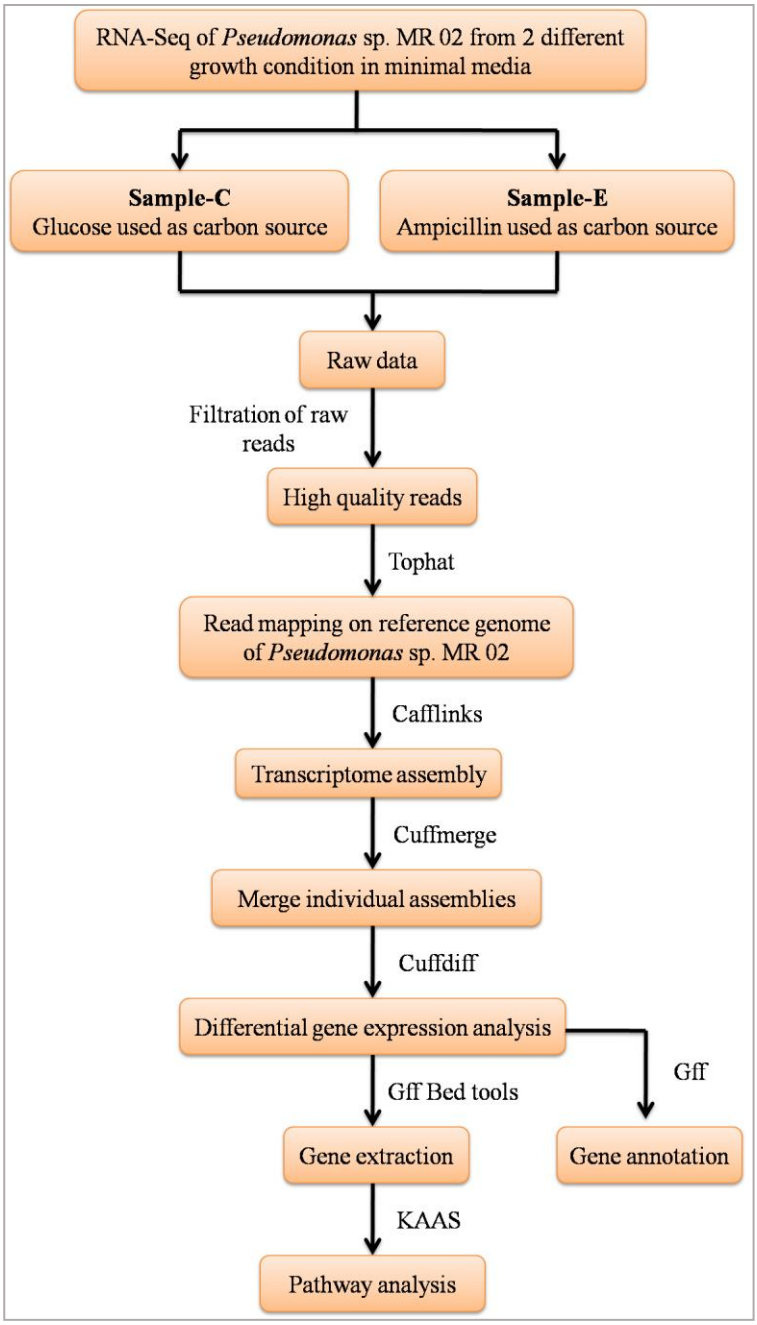

Figure 5.3: Overview of the RNA-Seq analysis steps of *Pseudomonas* sp. MR 02

5.2.4.2 Total RNA isolation, qualitative and quantitative analysis

Total RNA was isolated from both the samples (control 'C' and experiment 'E') using Quick-RNA Miniprep Plus total RNA isolation kit (Zymo Research) by following the standard protocol as described by the manufacturer. Total RNA was dissolved in nuclease-free water and the qualities/purity of RNA was checked by measuring Optical

Density Absorption ratio ($A_{260/280}$) using nanodrop and quantities of the isolated RNA were checked using 1% RNA agarose gel and visualization under U.V. light.

5.2.4.3 cDNA library preparation and sequencing

The paired-end cDNA sequencing libraries were prepared using total RNA per sample using a TrueSeq RNA sample preparation V2 kit (Illumina, San Diego California, USA) as per manufacturer's protocol. Then the cDNA fragments were purified with AMPure XP system and to select a fragment of 150-200bp in length for sequencing. The library quality was checked on Agilent 4200 bioanalyzer using High sensitivity DNA chip. For next-generation sequencing of both samples was performed using paired-end 2×150 bp library of the Next Seq 500 platform.

5.2.4.4 Preprocessing RNA-Seq data and alignment to the reference genome

The raw data of both samples were filtered using Trimmonatic v0.38 and high quality reads were obtained by removing the adapter and low-quality sequences from the raw data. The sequence reads were aligned to reference genome of *Pseudomonas* MR 02 using Top-hat V2.1.1 with default parameters (Trapnell *et. al*., 2009).

The reference genome of *Pseudomonas* sp. MR 02, with a genome size of ~5.9 Mb and the associated annotations were downloaded from NCBI Genome DB (https://www.ncbi.nlm.nih.gov/genome/13508?genome_assembly_id=354794).

5.2.4.5 Differential Gene Expression (DGEs) analysis

Assembled all transcriptome data from RNA-Seq data with help of cufflinks V2.2.1 program and quantifies their expression. The individual gtf files of the assembled transcriptomes were used for differential gene expression analysis using cuffdiff program. There are a total of 5,436 coding genes present in the annotation file of *Pseudomonas* sp. MR 02. Differential gene expression analysis was performed using cuffdiff v2.2.1 to obtain significantly differently expressed genes between control and experiment samples (Trapnell *et. al*., 2013). The analysis was carried out for commonly expressed genes reported between control and experiment samples respectively. FPKM values were used to calculate the log fold change as log2 (FPKM-experimental/ FPKM-control). Log2 fold change values greater than zero were considered up-regulated whereas less than zero were down-relegated along with *P*-value (≥0.05) for statistically significant results.

An average linkage hierarchical cluster analysis was performed on top 50 differentially expressed genes, of the differential genes, expressed combination control vs. experiment, using multiple experiments viewer (MeV v4.9.0) (Eheanor *et. al*., 2011). The heatmap shows the level of gene abundance. Levels of expression are represented as the log2 ratio of gene abundance between control and experiment samples. Differentially expressed genes were analyzed by hierarchical clustering. Heatmaps were created using the log-transformed and normalized value of genes based on Pearson uncentered distance and average linkage method.

5.2.4.6 Pathway analysis of coding DNA sequences by KEGG

The functional annotations of genes were carried out against the curated KEGG GENES database using KAAS ver. 1.6 (KEGG Automatic Annotation Server-. (http://www.genome.jp/kegg/ko.html). The KEGG Orthology database of Prokaryotes family was used as the reference for pathway mapping. The result contains KO (KEGG Orthology) assignments and automatically generated KEGG pathways using KAAS BBH (bidirectional best hit) method against the available database with default parameters (Moriya *et. al*., 2007).

5.2.5 Growth studies of MBL-positive strains to demonstrate utilization of ampicillin as sole source of carbon and energy

To examine the growth of four MBL-positive isolates of the *Proteus*, *Escherichia*, *Pseudomonas*, and *Acinetobacter* genera termed KR01, KR23, KR25 and MR40 (one isolate from each genus) in mineral medium supplemented with ampicillin as a sole source of carbon and energy, the inoculums and minimal media were prepared as following method described above section 5.2.1. Each culture was washed two times in distilled water lacking a carbon source by cell pellet centrifugation (3000 rpm, 5 min). After the final wash, the 10^6 cell suspension was resuspended in 100 ml Erlenmeyer flasks containing 20 ml minimal media with different supplementations. The basal minimal medium, used in this study, contained (g/L): KH2PO4, 3.0; Na2HPO4, 6.0; NaCl, 5.0; and MgSO4, 0.1(pH 7.0 ± 0.2). Ampicillin was used as the carbon source and the concentrations of ampicillin used were 0.5 g/L, 1g/L, 2g/L, and 4g/L. Flasks were incubated at 30ºC for 216 hrs in a shaking incubator at 120 rpm. All growth experiments

included triplicate independent cultures, and OD600 values were evaluated as averages with standard error. Growth data were plotted using Microsoft Excel.

5.3 Results and Discussion

5.3.1 Growth of MR 02 in minimal medium supplemented with ampicillin as a sole source of carbon, nitrogen, and energy in the presence or absence of EDTA

The strain *Pseudomonas* sp. MR 02 isolated from Mahananda River water, before growth study in minimal media with ampicillin as a carbon and nitrogen source and was found to be extensively antibiotic-resistant and it has been established as a pandrug resistance bacterial strain in previous chapter 4. We cultured strain MR 02 with aeration in minimal media containing 2 g/L, 4 g/L and 6 g/L ampicillin as the sole carbon and nitrogen source at 30°C and measured culture density over the 216 hrs (Figure 5.4). The strain *Pseudomonas* sp. MR 02 grew robustly in minimal media supplemented with 6 g/L ampicillin as the sole source of carbon (Figure 5.4 b). MR 02 growth was also observed when we used ampicillin used as both carbon and nitrogen source (Figure 5.4a). The strain MR 02 grow in a specific concentration (nearly half of the MIC value), they do not grow in a dose-dependent manner in both conditions (ampicillin used as an only carbon source or both carbon or nitrogen source), and the growth was observed only in 6 g/L ampicillin. MR 02 cells failed to survive when we added 0.4 mM EDTA (metal chelating agents) in minimal media and the same concentration of ampicillin used as nitrogen (Figure 5.4c) and carbon source (Figure 5.4d). It was found that in minimal medium containing glucose (8.0 g/L) and NH4Cl (2.0 g/L), growth was almost unaffected in the presence of 0.4 mM EDTA (Figure 5.5).

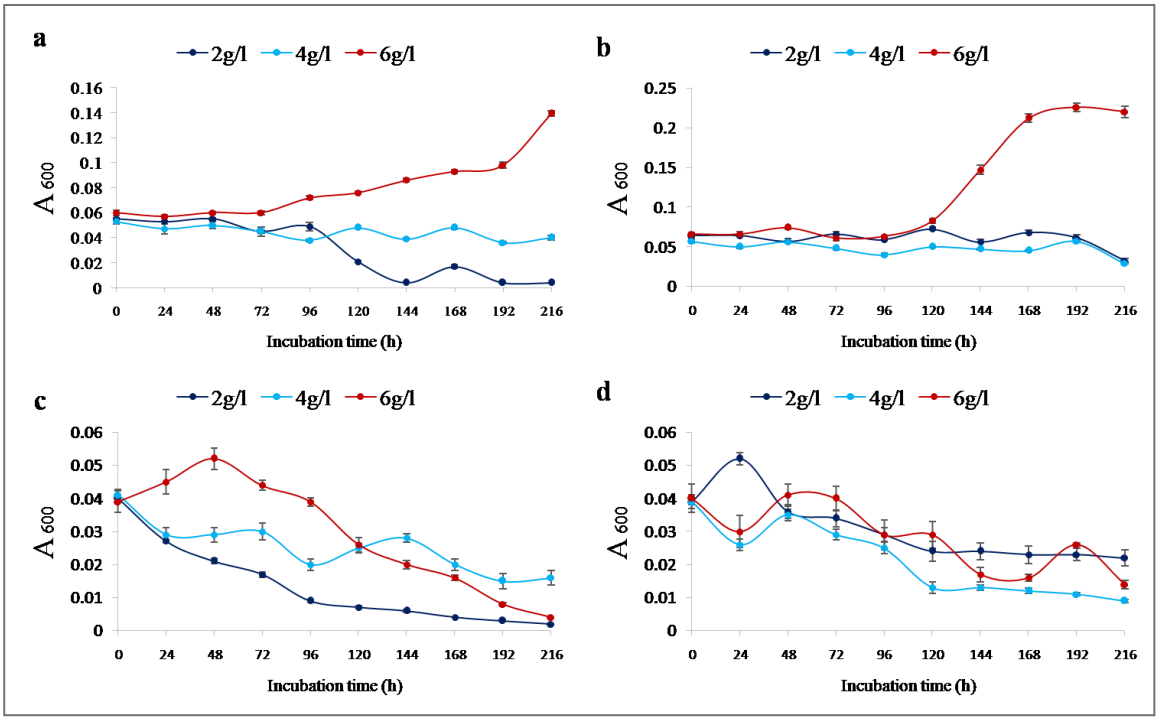

Figure 5.4(a-d): Consumption of ampicillin as a growth substrate by *Pseudomonas* sp. MR 02

Pseudomonas sp. MR 02 used β-lactam as a sole carbon and nitrogen source for growth (a) Growth of MR 02 in the presences of ampicillin as a both sole carbon and nitrogen source (b) Growth of MR 02 in the presences of ampicillin as a only carbon source (c) Growth of MR 02 in the presences of ampicillin as a both sole carbon and nitrogen source with Metallo-β-lactamase Sub-inhibitory concentrations of EDTA (0.4 mM) (d) Growth of MR 02 in the presences of ampicillin as a only carbon source with Metallo-β-lactamase Sub-inhibitory concentrations of EDTA (0.4 mM).

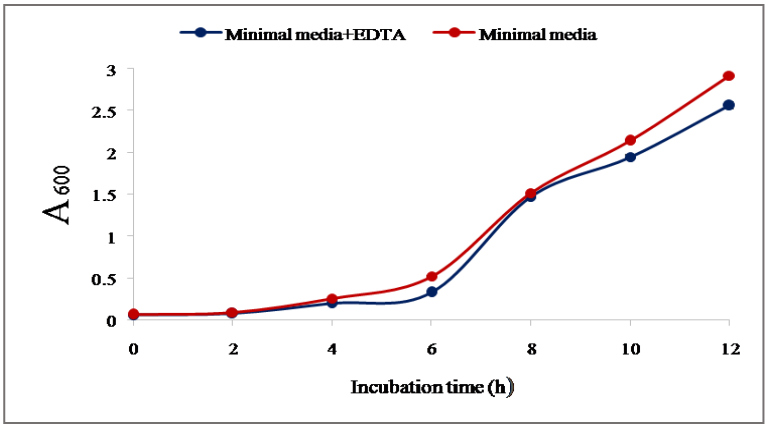

Figure 5.5: Growth of *Pseudomonas* sp. MR 02 in minimal media in the presences or absence of EDTA (0.4 mM EDTA) as a control

5.3.2 Identification of β-lactam catabolism responsible gene

We have an annotated draft genome of MR 02 to explicate the enzymes and pathways utilized during β-lactam catabolism. We annotated three chromosomal encoded β-lactamase genes belonging to class A extended-spectrum β-lactamase *bla*$_{PME-1}$, subclass B1 Metallo-β-lactamase *bla*$_{NDM-1}$, and class C β-lactamase AMPC, genes belonging to structural genes for amidase operon and also genes for the complete phenylacetic acid catabolic pathway. A figure has been constructed with the annotated genes and operons for catabolism of β-lactam antibiotic, ampicillin (Figure 5.6).

Hence, we hypothesize that *Pseudomonas* sp. MR 02 catabolizes ampicillin in following steps: (i) Cleavage of the β-lactam ring and remove an amino group of ampicillin by NDM-1 (ii) amidase enzyme cleaves amide bond of inactivated ampicillin and releasing phenylacetic acid and ammonia molecules (NH_4^+) (iii) phenylacetic acid is routed to central metabolism as a carbon source by the phenylacetic acid catabolic pathway and (iv) two ammonia molecules (NH_4^+) which is routed to central metabolism use as a nitrogen source for amino acid synthesis and other reduced nitrogen-carbon compounds synthesis (Figure 5.7).

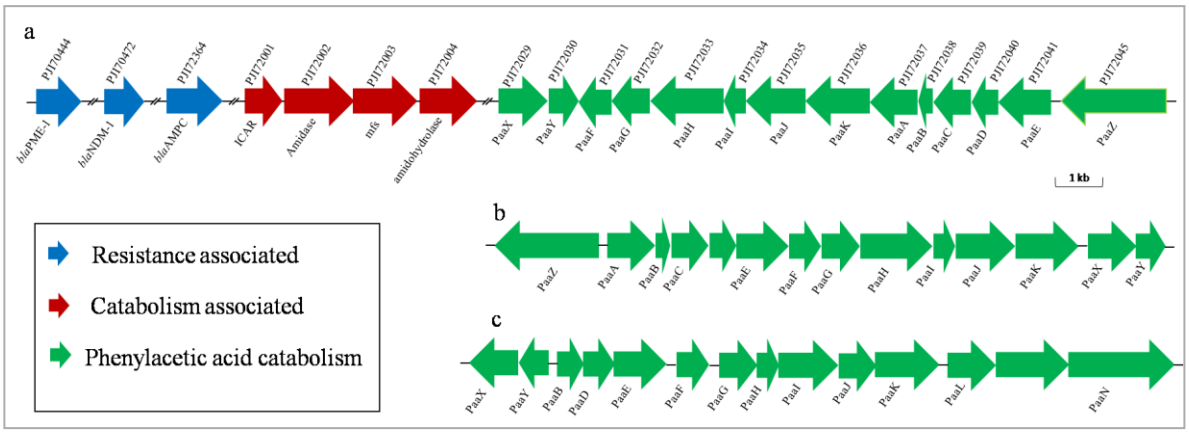

Figure 5.6: Annotations of genes responsible for ampicillin catabolism

(a) Genes and ORF responsible for β-lactam catabolism in *Pseudomonas* sp. MR 02; blue colour indicating genes coding for β-lactamases; red colour indicating genes for amidase operon; and green colour indicating genes for phenylacetate degradation; (b) Complete ORF of phenylacetate degradation in *E. coli* K12; (c) Complete ORF of phenylacetate degradation in *Pseudomonas* sp. PE-S1G-1.

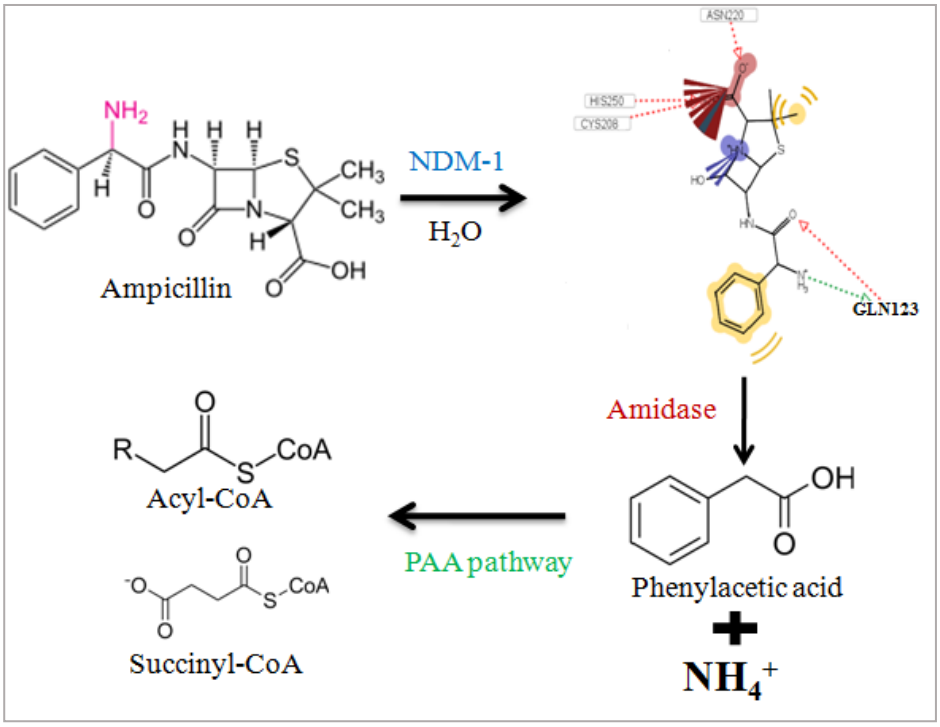

Figure 5.7: Hypothesized pathway for ampicillin catabolism

(Docking image was adopted from Wang *et. al*., 2015)

5.3.3 *E.coli* expressing MR 02 *bla*$_{NDM-1}$ catabolizes ampicillin

Based on our growth studies of MR 02 in minimal medium supplemented with ampicillin as carbon and nitrogen source in presence and absence of EDTA (0.4 mM), we hypothesized an additional attribute of NDM-1 to support the growth of any bacterium to utilize ampicillin as carbon and energy source, provided it has dedicated amidase(s) and the pathway to catabolise phenyl acetic acid (PAA). To determine the necessity of NDM-1 in ampicillin catabolism, the recombinant *E. coli* DH5α cells were used because it having a similar metabolic pathway. Hence the *bla*$_{NDM-1}$ gene of *Pseudomonas* sp. MR 02 was cloned and expressed in *E. coli* DH5α cell. Before the growth study first, we determine the MIC value of recombinant *E. coli* DH5α + NDM-1 strain (Table 5.1). Recombinant *E. coli* DH5α cells showed ampicillin resistance more than or equivalent 256 µg/ml. The recombinant *E. coli* DH5α+ NDM-1 strain was able to grow in ampicillin (100 mg/L) as sole carbon and energy source (Figure 5.6). There by supporting the hypothesis to prove

the associated role of *bla*$_{NDM-1}$ in ampicillin catabolism. Two different recombinant strains E. coli DH5α_pHSG398 and E. coli DH5α_pGEM-T were used as controls (Figure 5.6).

Table 5.1: Antimicrobial susceptibility pattern of *E.coli* DH5α:pHSG398 and *E.coli* DH5α:pHSG398+NDM-1

	MIC (µg/ml)					
	Ampicillin	**Penicillin**	**Imipenem**	**Meropenem**	**Cefepime**	**Ceftazidime**
DH5α+ pHSG398	6	6	0.45	0.9	0.02	0.09
DH5α+ pHSG398/ *bla*$_{NDM-1}$	≥256	≥256	4	16	32	32

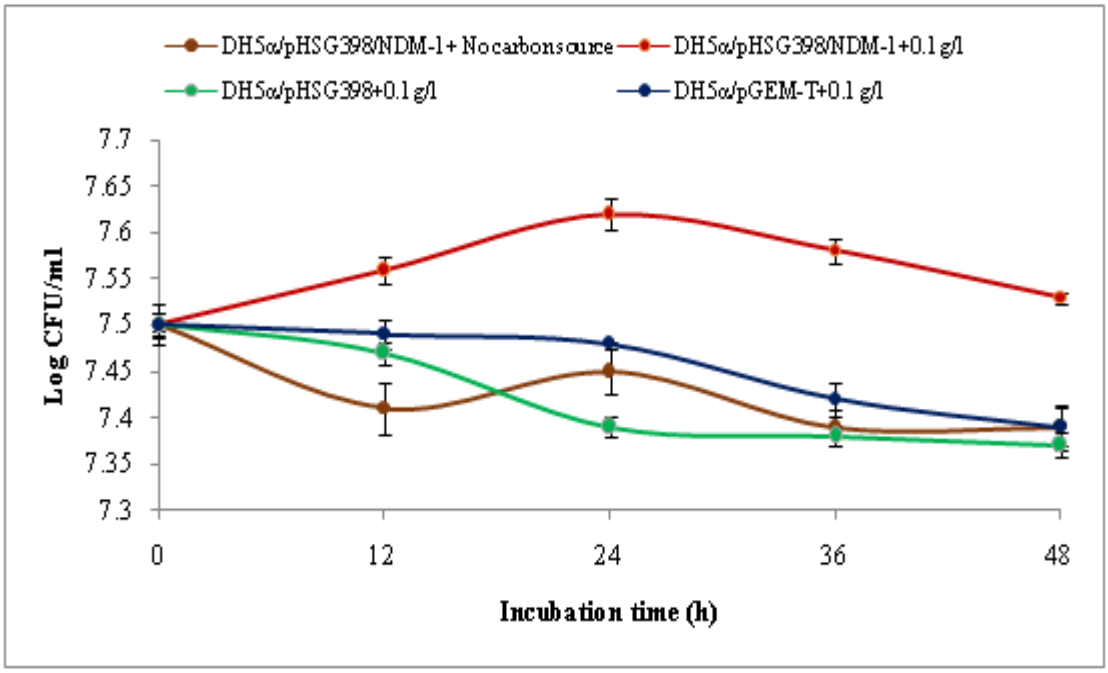

Figure 5.8: Growth of recombinant strain, *E. coli* DH5α: pHSG398+NDM-1 in minimal media with ampicillin as sole carbon

Recombinant strain DH5α/pHSG398 and DH5α/pGEM-T used as negative control and positive control respectively. Data points are average of three experiments with standard error bars. OD_{600} Optical density at 600 nm.

5.3.4 Whole transcriptome sequencing of *Pseudomonas* sp. MR 02

5.3.4.1 Qualitative and quantitative analysis of RNA samples

Qualitative analysis of the total RNA of both control and experiment cells of *Pseudomonas* sp. MR 02 was resolved on 1% agarose gel at 80V for approximately 60 min till sample reached 3/4th of the gel (Appendix A5.1) and 1 µl of RNA of both sample was loaded in nanodrop for quantitative analysis. The quality control (QC) passed samples were used to prepare cDNA library.

5.3.4.2 RNA sequencing, transcriptome assembly and aligning to the reference genome

Two cDNA libraries of *Pseudomonas* sp. MR 02 cultured in minimal media with glucose as carbon source and minimal media with ampicillin as carbon source were sequenced on Illumina Next Seq 500 platform and name as *Pseudomonas* sp. MR 02 control 'C' and *Pseudomonas* sp. MR 02 experiment 'E' respectively. There were a total of 9,211,961 and 8,035,277 raw reads were generated comprising of 2,752,874,653 and 2,400,906,916 nucleotide bases in control and experiment libraries, respectively (Table 5.2). After the quality filtration (mean quality score < 20 Phred score) and adaptor trimming using Trimmomatic v0.38, the high-quality reads were used for reference-based read mapping. The raw reads were submitted to the NCBI database under BioProject number PRJNA612657 with BioSample numbers SAMN14379771 and SAMN14379772. As the draft genome sequence of *Pseudomonas* sp. MR 02 strain has been published, the sequence reads were then aligned to the database for further analysis of gene expression profile. More than 94% of the total reads of both samples could be aligned to the *Pseudomonas* sp. MR 02 draft genome, of which approximately 99% could be identified read mapped and used for gene expression analysis. In taxonomy analysis 63% and 37% reads were aligned, control sample read and experiment sample read with MR 02 draft genome respectively. After genome mapping 3721 and 3710 genes were detected to be expressed in control and experiment respectively with *Pseudomonas* sp. MR 02 respectively. Among these genes, 3707 were commonly expressed between both samples.

5.3.4.3 Differential genes expression analysis

Differential gene expression profile between carbon source as glucose and carbon source as ampicillin transcripts was developed using cuffdiff v2.2.1 to identify genes with differential expression level in the control compared to experiment, initially we used the FPKM method to calculate the expression level of the CDS. Differentially expressed genes were analyzed by hierarchical clustering. A heat map was constructed using the log transformed and normalized value of genes based on person uncentered distance as well as based on average linkage method.

Table 5.2: Differential genes expression summary

Description	Gene counts				
	Upregulated	**Downregulated**	**Exclusive only in control sample**	**Exclusive only in experiment sample**	**Expressed in both**
***Pseudomonas* sp. MR 02, Control vs. Experiment**	93	45	14	3	3707

Based on the common hit accession of functionally annotated CDS in control and expressed strain CDS, a total of 3306 CDS expressing in both sample transcripts of which a total 45 (1.21%) CDS were down-regulated in control strain transcript compare to experiment strain transcript whereas 93 (2.50%) CDS were up-regulated in control compared to experiment with the log 2 fold change value of greater than zero (Table 5.2).

Heatmap was constructed on the average linkage hierarchical cluster analysis, shows the levels of transcript abundance of the top 50 differentially expressed transcripts recognized in control and experiment samples. The heat map represents that out of 50 genes, nearly 50% of the genes were shown to be upregulated and the rest 50% nearly to be down regulated. Among these 50 genes, metallo-β-lactamase NDM-1 gene is present (Figure 5.8). The red and green color in the heat map represent (logarithmic intensity of the expression genes) upregulated and downregulated genes respectively, among the top 50 differentially expressed genes in both control and experiment samples.

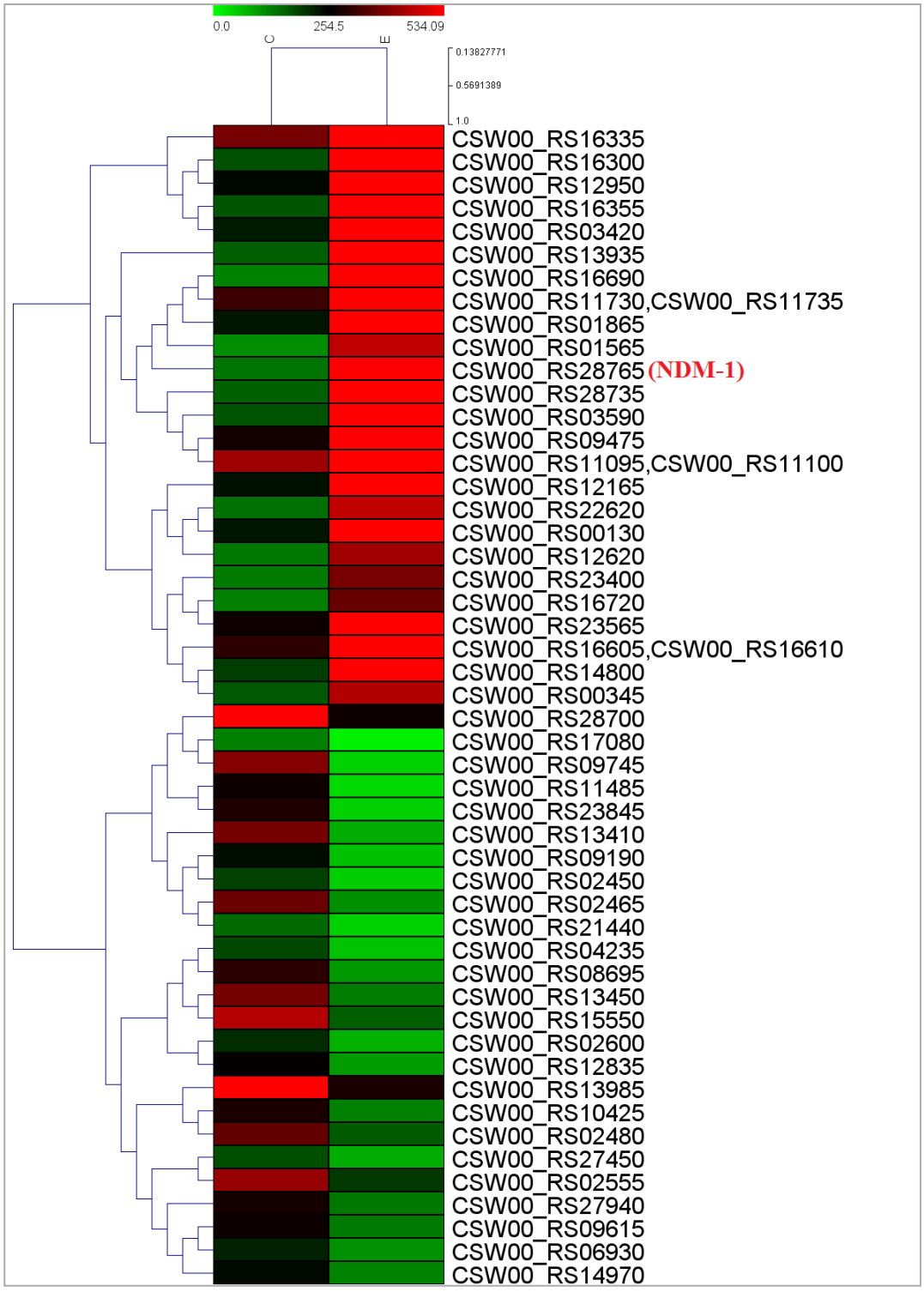

Figure 5.9: Heatmap of top 50 differentially expressed genes of *Pseudomonas* sp. MR 02 grown in glucose and ampicillin as carbon source containing minimal media

5.3.4.4 KEGG pathway analysis of DEGs

Gene ontology enrichment analysis was performed with the 93 CDS up-regulated in control compared to experiment (Appendix A5.1). This study was focused on ampicillin catabolism, how bacteria use ampicillin as carbon source and this is also shown in transcriptome study. The genes identified were found to be classified into 21 functional pathway categories in KEGG. Most of the annotated genes were found to associate with carbohydrate metabolism (138) energy metabolism, lipid metabolism, signal transduction and environmental adaptation (Table 5.3).

Table 5.3: KEGG pathway classification

Pathways	**Gene counts in C vs. E**
Metabolism	
Carbohydrate metabolism	136
Energy metabolism	90
Lipid metabolism	57
Nucleotide metabolism	42
Amino acid metabolism	181
Metabolism of other amino acids	57
Glycan biosynthesis and metabolism	27
Metabolism of cofactors and vitamins	85
Metabolism of terpenoids and polyketides	22
Biosynthesis of other secondary metabolites	28
Xenobiotics biodegradation and metabolism	49
Genetic Information and Processing	
Translation	20
Folding, sorting and degradation	15
Replication and repair	22
Environmental Information and Processing	

Membrane transport	136
Signal transduction	159
Cellular Process	
Transport and catabolism	9
Cell growth and death	17
Cellular community- prokaryotes	92
Cell motility	37
Organism System	
Environmental adaptation	11

5.3.4.5 Study of functional genes in ampicillin catabolism

According to our hypothesis the ampicillin catabolism required three different components (i) metallo-β-lactamase *bla*$_{NDM}$ genes (ii) amidase genes and (iii) PAA pathway genes. Based on the comparative RNA-Seq it was demonstrated that the expression of ampicillin catabolism responsible genes coding for NDM-1, amidase and PAA pathway, there has been a change in the expression of metabolic enzymes to presence of growth requirements in minimal media containing ampicillin as sole carbon source. We identified that of the 138 differentially expressed genes, 93 and 45 genes were significantly upregulated (Table A5.1) and downregulated (Table A5.2) respectively (Figure 5.8), in MR 02 cells when grown in minimal medium containing ampicillin as sole carbon and energy source compared to the cells grown in minimal medium containing glucose as sole carbon and energy source. The expression of B1 metallo-β-lactamase NDM-1 gene was significantly higher (upregulated; $\log_2$ fold change of 2.00088) than two other β-lactamases, class A extended-spectrum β-lactamase PME-1 and PDC (β-lactamase class C) respectively, in ampicillin-grown cells compared to glucose-grown cells. Two genes, coding for penicillin G amidase and penicillin acylase family protein respectively; and three genes coding for MBL fold metallo-hydrolases; and all paa genes of the phenylacetic acid catabolism were expressed in both conditions. On the basis of the comparative transcriptomics, it was revealed that there has been alteration of the expression of metabolic genes to meet growth requirements in minimal medium containing ampicillin as sole carbon and energy source.

5.3.5 Growth studies of MBL-positive strain in minimal-medium supplemented with ampicillin as sole source of carbon

Four MBL positive strains one from each genus, *Proteus* sp. KR01, *Escherichia* sp. KR23, *Pseudomonas mendocina*. KR25 and *Acinetobacter* sp. MR40, were selected to test viability and growth in minimal media supplemented with ampicillin as a sole source of carbon. Among these four strains, *Escherichia* sp. KR23 carry $bla_{\text{NDM-7}}$ genes, *Pseudomonas mendocina* KR25 carry $bla_{\text{VIM-2}}$ and other two strains carry $bla_{\text{NDM-1}}$ genes. The concentrations of ampicillin used in this study were 1.0 g/L, 2.0 g/L, and 4.0 g/L to support the growth of KR01, KR23 and KR25 and 0.5 g/L, 1.0 g/L, and 2.0 g/L ampicillin for MR40 (Figure 5.10). The cells of *Proteus* sp. KR01 has demonstrated the viability till 72 h followed by marginal cryptic growth (similar to growth that happens during stationary phase) but no visible growth showing persistent log phase (similar to a typical growth curve) took place (Figure 5.10a). In case of *Pseudomonas medocina* KR25 has shown the viability till 196 h followed by marginal cryptic growth at only 1 g/L ampicillin concentration but no growth was observed in any concentration (Fig. 5.10c). Of the different concentrations of ampicillin used as sole carbon and energy source to test growth, *Escherichia* sp. KR23, and *Acinetobacter* sp. MR40 demonstrated maximum growth in 4.0, and 1.0 g/L ampicillin respectively (Figure 5.10 b & d). Each strain grew in only one ampicillin concentrations (nearly half of the MIC value). These results confirm the validity of our hypothesis NDM-1 or NDM-7 role in ampicillin catabolism.

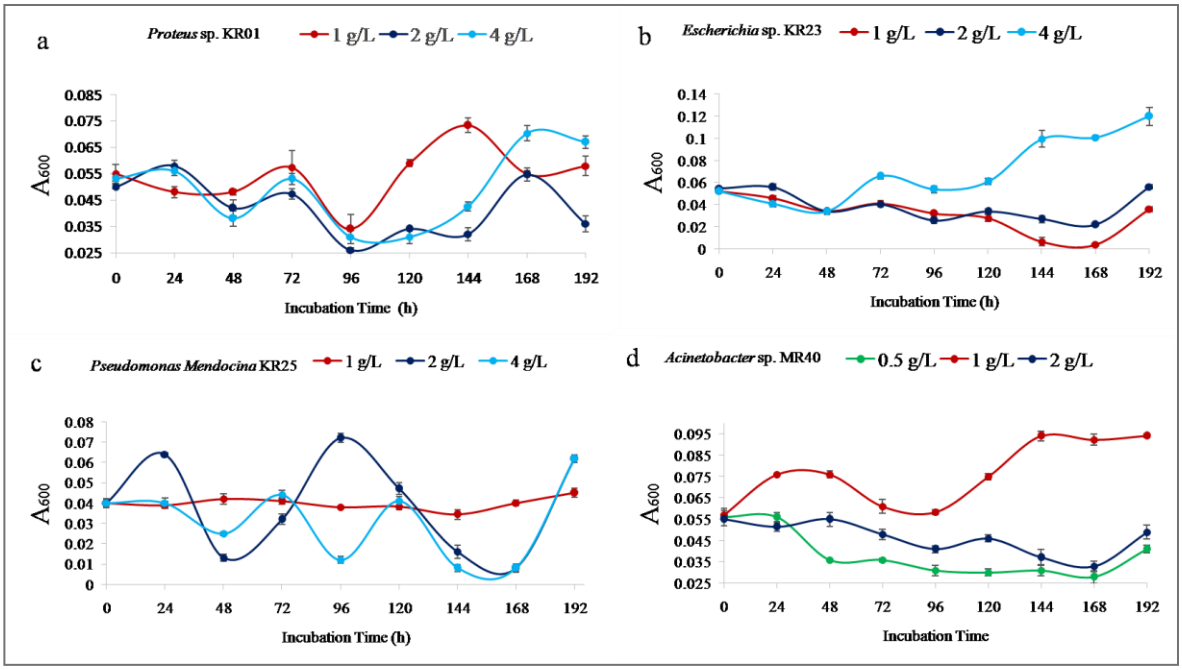

Figure 5.10: MBL positive strains catabolise ampicillin and use as sole carbon source a-d,

(a) growth of strain *Proteus* sp. KR01, (b) growth of strain *Escherichia* sp. KR23, (c) *Pseudomonas medocina* KR25, (d) *Acinetobacter* sp. MR40, in minimal media with ampicillin as sole carbon source. Data points are average of three experiments with standard error bars. OD_{600} Optical density at 600 nm.

5.4 Conclusion

Antibiotics resistance enzymes are known to be plentiful in soil habitats, and it is only because of their medical exploitation that as privileged molecules not bound by the carbon cycle. Gram-negative NDM-1 producers of the genera, *Acinetobacter*, *Escherichia*, and *Pseudomonas*, demonstrating significantly high MIC values against β- lactam antibiotics, endowed with the capacity of consuming ampicillin as a carbon-source has larger implications in cleaning antibiotic-stressed habitats.

General Discussion

Carbapenems are a broad range of β-lactam antibiotics currently considered to be the drug of 'last resort' for treating infections with multi-drug resistant bacteria. In the past two decades, the development and spread of strains resistant to carbapenem have been a growing concern (Meletis, 2016). Imipenem and meropenem are common carbapenem antimicrobial agents used to treat nosocomial and mixed bacterial infections. Resistance to these antimicrobial agents limits therapeutic options. Resistance to carbapenems occur either through bacterial production of metallo-β-lactamase enzymes that hydrolyze the carbapenems, and break down other β-lactam antibiotics as well (Queenan and Bush, 2007) or through porin channels or efflux pumps in the bacterial cell wall that reduce the permeability of the drug into the organism or efflux the drugs from organism (Fernandez and Hancock, 2012). In some bacteria like *Pseudomonas aeruginosa*, all these three mechanisms are present (Fernandez and Hancock, 2012). The most dangerous bacteria are known to produce metallo-β-lactamase enzymes and can pass it from organism to organism via plasmid or integron and a reservoir is required for horizontal gene transfer (Lutgring and Limbago, 2016; Meletis, 2016; Diene and Rolain, 2014). Human and animal gut, natural environment, including soil and water are the potential reservoir of antibiotic resistance genes. Antibiotic resistance is often focused around health sectors and the hospital environment has been shown to harbor and distribute antibiotic resistance including metallo- β-lactamase (Bush *et. al*., 2011). Metallo-β-lactamase producing bacterial strains is mainly reported in a hospital setting and these pathogenic bacterial strains release to environment from hospital sewage via WWTPs and waterways (Ray *et. al*., 2016; Zenati *et. al*., 2016).

The primary aim of this research was to investigate the presence and abundance of MBL producing bacteria in two city-waste polluted rivers. Since the resistance level of bacteria is settled on by the enzyme efficiency for hydrolyzing carbapenems and by the number of resistance mechanisms present in the bacteria, and by other probabilities through production of more than one hydrolyzing enzyme and may show modifications in more than one porin, producing high-level resistance to the carbapenemase as per German DIN 58940 standard (1998). The concentration of imipenem prescribed in media for isolation of imipenem-resistant bacteria is 8 μg/ml, we used 20 μg/ml imipenem in MHA

plates, in this study, to determine the density of imipenem-resistant bacteria in river water sample. We have also considered breakpoint criteria (S/R) changes for imipenem when testing *Enterobacteriaceae* while selecting 20 µg/ml (as in CLSI and EUCAST guidelines imipenem resistant is > 8 µg/ml) imipenem concentration to select isolates bearing MBLs (https://clsi.org/; http://www.eucast.org/clinicalbreakpoints/). Moreover, the level of resistance is determined by the efficiency of the enzyme for hydrolyzing the drug and by the number of resistance mechanisms present in the organism. Organisms can produce more than one hydrolyzing enzyme and may show modifications in more than one porin, producing high –level resistance to the carbapenems (MIC ≥ 16 µg/ml); while organisms with decreased susceptibility produced by pouring changes alone often have lower MICs (2 -8 µg/ml). Therefore, if we had used 8 µg/ml imipenem, we might have ended with an overwhelming high number of intrinsically imipenem resistant isolates. Earlier authors have also reported about the low prevalence of carbapenem-resistant bacteria in river water; intrinsic resistance being pre-dominant (Farzana *et.al*. 2013).

The water quality data of the two rivers, Mahananda and Karala, indicated that both of them are moderately polluted (unpolluted rivers typically have a BOD below 1 mg/L) (Appendix D1). The BOD was measured in the range of 1.6 – 2.1 mg /L in water samples collected from the rivers in the March and April (Pre-monsoon months), November and December (post monsoon months) of the year 2015. The dissolved oxygen (DO) is an important parameter to monitor the biological quality of water as it supports the aquatic life forms and regulates the biological degradation of organic impurities. In the pre-monsoon months, DO in the month of April, for both the rivers, was found to be lower than March data; while in the post-monsoon months, DO recorded in the month of November was lower than December (Table. D1). A slight decrease in DO at sampling location could be attributed to the addition of sewage upstream. The amounts of chloride in a water sample can give an indication of the amount of sewage effluent in river water. The chloride content of the Mahananda and Karala River water in the month of April was 1.9 and 2.7 times respectively, the content recorded in the March (Table. D1). Again, in the post-monsoon months, the chloride content in the November of both Mahananda and Karala was higher than the value obtained in the December. The main source of chlorides in the

waters is the discharge of domestic sewage; therefore, its concentration serves as an indicator of pollution by sewage.

The physiochemical data corroborated with the microbiological data on imipenem-resistant bacteria present in the water samples collected in March and April (pre-monsoon months) and, November and December (post-monsoon months). Data confirms maximum load of imipenem-resistant bacteria in the mid and downstream of both rivers in the April and November. Among water samples collected in December, from all the three sampling stations of the two rivers, imipenem-resistant bacteria were comparatively more in the downstream of Karala. The upstream of Karala River is situated in the less-populated rural region while it veins and bisects the town in two halves; the midstream is at the heart of the town very near to the District hospital, but in post-monsoon months maximum stagnation occurs in its downstream; this may explain the abundance of imipenem-resistant bacteria in the downstream water samples. Of the total number of imipenem-resistant bacteria retrieved from Mahananda River, maximum were derived from the midstream water sample followed by the downstream; least numbers were obtained from upstream water samples. This could be because all municipal waste channels carrying community and hospital waste-water are finally discharged in the mid-stream. The upstream and downstream of Mahananda drains predominantly less populated rural setting and also fast-flowing (due to persistent gradient) and less stagnating in pre-and post-monsoon months compared to Karala. Huge quantities of antibiotics are discharged into municipal waste because of the partial metabolism of humans or due to removal of idle antibiotics ensuing in the detection of antibiotic-resistant bacteria and ARGs (Zhang *et. al*., 2009, Selvaraj *et. al*., 2013, Rutgersson *et. al*., 2014, Chakraborty *et. al*, 2013). Hospital wastewaters contain fecal material from a large number of individuals, of which many are undergoing antibiotic therapy. It is, thus, conceivable that city-waste polluted river waters could provide opportunities to find novel carbapenemases and other resistance genes not yet described in clinical strains.

The most common metallo-β-lactamase detected in the both rivers isolates were bla_{NDM} (93.4%). The percentage occurrence of MBL-positive isolates among imipenem-resistant bacteria isolated from Mahananda and Karala River was 5.26% and 4.68% respectively. The gene, bla_{NDM}, was detected in 17.14% and 20.51 % of MBL-positive

strains isolated from Mahananda and Karala River respectively. Large number of imipenem-resistant bacteria, isolated from waters of the Mahananda and Karala river, which were not MBL-positive ones, could be those who have recruited other mechanisms like over expression of efflux pumps leading to carbapenem resistance associated with multi-drug resistance (Meletis *et. al*., 2012), or are able to prevent carbapenems reaching their PBPs by diminishing the permeability of their outer membrane by diminished expression or loss of specific porins, or mutation derived changes of their PBPs as in gram positive bacteria (Munita & Arias, 2016).

Bacterial resistance to antibiotic molecule, cover almost all clinically used antibiotic classes, and is leading the world towards a return to the condition of the pre antibiotic era (Fair and Tor, 2014). Antibiotic susceptibility testing and MIC results of all 15 MBL producing strains against 22 antibiotics (Table 1. 7 and Table 1.8) and 13 antibiotics (Table 1.9 and Table 1.10) respectively, showing that all MBL producing strain are multidrug-resistance and in this study we have established one MBL producing strain MR 02 as PDR (Pan-drug resistance) strain because both phenotypically and genotypically, it has shown resistance against all available clinically used antibiotics. In the current study, the MIC value vary between same bacterial species, Karala River MBL producing isolates KR07, KR22 and KR23 are belonging to same bacterial species but they are showing different MIC value against different antibiotics, similarly this type phenomenon happen with Mahananda River MBL producing isolates MR40 and MR41, both strain also belonging same bacterial species but MIC values are different. In this study we also characterised integron gene cassettes in MBL positive isolates, and found that the same species but different bacterial strain that was isolated from the same sampling site carried different antibiotics resistance gene cassettes. This result indicates that polluted river water is the hotspot of resistance evolution.

While identifying metallo-β-lactamase activity in this current study (Fig 1.10 and Fig 1.11) it was notated that isolates MR 02, MR15, MR40, MR41, KR01, KR04, KR07, KR22 and KR23 produced more than one class of β-lactamase. Same finding was also observed in some recent study and it was found that two different classes of β-lactamase gene MBL and CTX-M type are present in same *Klebsiella pneumoniae* strain (isolate from hospital in Italy) (Nucleo *et. al*., 2013). The production of different type's β-

lactamase in same bacterial strain makes it more resistant to antibiotic classes (Extensively drug-resistant or Pan-drug), and producing offspring of resistant bacteria with much more detrimental effect upon morbidity and mortality of human beings (Nucleo *et. al*., 2013).

The second observation is the detection of high frequencies of multidrug resistant pattern among the river water MBL producing isolates which may be attributable to intrinsic resistance. It has been claimed that water and soil bacteria are the most important source of antibiotic resistance (WHO 2020 https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance).

16S rRNA gene being universal, 16S rRNA sequences are widely used to ascertain the genus and species of the isolates (Srinivasan *et. al*., 2015). After the isolation and screening of MBL producing isolates from both the river water samples, all of these isolates were identified by 16S rRNA gene sequencing and variation in bla_{NDM} genes were detected by PCR amplification and sequencing of the complete ORF region. Thus, the amplification and sequencing results of bla_{NDM} genes among the both rivers isolates showed similarities with $bla_{\text{NDM-1}}$ and $bla_{\text{NDM-7}}$ genes within GenBank. In this study, a large portion (07 out of 35, and 08 out of 39 from Mahananda and Karala respectively) of MBL-positive isolates, possessed NDM; and only one of the Karala isolates contained VIM-2 (Table 2.2). Fourteen bla_{NDM} possesing multidrug-resistant isolates belonged to five genera, *Pseudomonas*, *Myroides*, *Acinetobacter*, *Proteus*, and *Escherichia*; the only one isolate bearing $bla_{\text{VIM-2}}$ belonged to the genus *Pseudomonas* (Table 2.1). Three Karola river isolates, KR07, KR22, and KR23, bearing $bla_{\text{NDM-7}}$ belong to a novel species of *Escherichia* having closest phylogenetic relationship with *Escherichia albertii*. KR07 and KR22 contained one and two class 1 integrons respectively. Very recently, bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$ were detected in *E. albertii*, an emerging member of the *Enterobacteriaceae* causing human and animal enteric infections (Li *et. al*., 2018). The first novel NDM-7 was also identified in 2013 in *E. coli* bacterial strain and it isolated from urine culture in France (Cuzon *et. al*., 2013).

NDM has been detected in the river water in Brazil (South Africa); in drinking water in Canada (North America) and in the Danube River water samples in Europe, (Sanchez *et. al*., 2018; Fernando *et. al*., 2016; Kittinger *et. al*., 2016). This indicates the importance of the river water and the water environment as it contains numerous bacteria,

some of which are metallo-β-lactamase and these bacteria in river water or drinking water can originate from hospital waste, aquaculture and WWTPs (Li *et. al*., 2014).

Overwhelming abundance (40%) of the *bla*$_{NDM}$ gene was found in the both river MBL-positive isolates belonging to the genus *Acinetobacter*. Two Mahananda river NDM-1 positive isolates MR26 and MR29 belonging to one novel species; and KR26 to another novel species of *Acinetobacter* may be ascertained from the phylogenetic tree; one Karala River isolates, KR29, were found close to *A. seohaensis*; and the remaining two isolates of the genus *Acinetobacter*, MR40 and MR41, were close to *A. variabilis*. The isolates, MR40 and MR41, contained two class 1 integrons (one containing two gene cassettes, *aad*B-*aac*(6')-Ib; the other contains *dfr*A5 in MR40; in MR41, *bla*$_{PSE-1}$ present in one class 1 integron and *dfr*A5 in the other one). The knowledge of the occurrence and pattern of antimicrobial susceptibility pattern of *Acinetobacter* spp. is significant as they have come out as a cause of ICUs infection (Rebic *et. al*., 2018). Among the six most important nosocomial MDR pathogens, *A. baumannii* has been reported to cause 2-10% of all hospital-borne infections caused by Gram negative bacteria (Antunes *et. al*., 2014). The first report from India about NDM-1 in *Acinetobacter* was in the year 2012 (Karthikeyan *et. al*., 2012). Earlier authors have indicated a higher propensity of isolation of NDM-1 producing *A. baumannii* from sewages of the hospitals, but could not recover any NDM-1 producing isolates from a river or fish pond water (Zhang *et. al*., 2013). We have estimated about the possession of *bla*$_{NDM-1}$ in diverse species of the genus *Acinetobacter* in waters of the Mahananda and Karala.

On examining the water quality of both the rivers, it cannot be concluded that bacterial communities within it easily grown in nutrient-rich environment, moderately they are subjected close to oligotrophic condition. All the multidrug-resistant MBL-positive isolates were facultatively oligotrophic in nature. Facultative oligotrophs are less reactive to abruptive resource availability; instead they switch on to exploit nutrient poor conditions.

The increase in gram-negative carbapenem antibiotic resistance is worrisome particularly as there are few, if any clinical trial antimicrobial agents possessing suitable activity against *Pseudomonas* spp., *Acinetobacter* spp. and *Escherichia* spp. and then these bacterial species increase own resistance by acquiring the metallo-β-lactamase (MBL)

gene, which can potentially confer broad-spectrum β-lactam resistance including carbapenem. In several studies, MBL genes are often associated with aminoglycoside resistance genes and thus bacteria that possess resistance against carbapenem antibiotics as well as aminoglycoside antibiotics, both types of genes can be found as gene cassettes and it carried by mobile genetic element like integron (Walsh, 2005). Integron consist of three regions and among these one are called variable regions (fig 3.1) and within the variable region they can carry more than one gene. Gene encoding MBL, IMP and VIM are found as gene cassettes in class 1 integrons (Poirel *et. al*., 2001; Senda *et. al*., 1996; Yan *et. al*., 2001). One among the three multi-drug resistant MBL-positive *Pseudomonas* spp., KR25, was found to bear two class 1 integrons which included five gene cassettes (*bla*$_{\text{VIM-2}}$, *aac*A4, *aad*A1, *aad*A2, and *aac*(6′)-Ib) (Table 3.1 and Figure 3.4) and phylogenetically it nearest to *P. mendocina*. Other two *Pseudomonas* strains, MR15 and MR20, possessing *bla*$_{\text{NDM-1}}$, belong to *P.putida* lineage, closest to *P. monteilii* and *P. plecoglossicida*. In an earlier study from China, a considerable prevalence of class 1 integrons in multidrug resistant clinical *P. aeruginosa* isolates was observed (Chen *et. al*., 2009).

In the recent past, aquatic sediments receiving partially untreated effluents from communities of two different countries, Congo and India of diverse climatic conditions, were investigated for the occurrence of MBL genes in *Pseudomonas* spp.; and revealed that *bla*$_{\text{VIM-1}}$ in congo isolates but to a lesser degree in Indian isolates, but incidence of *bla*$_{\text{NDM-1}}$ was highest among the Indian isolates and few from Switzerland isolates (Devarajan *et. al*., 2017).

Out of a total of 19 class 1 integron borne gene cassettes, the most abundant (09/19) was *aad*A family of genes which encode aminoglycoside-3-adenylyltransferases (AAD) which encodes streptomycin and spectinomycin resistance by adenylation (enzyme modification), and the second most common (04/19) was *aac* (6′) Ib gene which code for aminoglycoside 6′-N-acetyltransferase type I b, an enzyme of clinical importance that catalyzes the transfer of an acetyl group from acetyl CoA to the 6′-amino group of aminoglycoside molecules conferring amikacin and kanamycin resistance and it found in a wide variety of gram-negative pathogens.

The abundance of *aad*A genes in Mahananda and Karala rivers of northern West Bengal, India, could be due to the rampant use of streptomycin in agriculture and they

encode kanamycin, neomycin, amikacin and streptomycin resistance (Bennett, 1999). Paddy and tea are the main cash crops and amongst horticultural crops, citrus fruits, tomato, and potato are widely grown in this region. The formulation of streptomycin sulphate + tetracycline hydrochloride (9:1) (is one of the antimicrobials registered in India as fungicide and bactericide as per insecticide act, 1968; sold as 'Antibac' trade-name;) is sprayed in huge quantities (for example to protect paddy from bacterial leaf blight, 300 g/ha is sprayed) to control blister blight in tea, bacterial leaf blight of paddy, citrus canker in citrus, brown wilt, or ring or bangle disease of potato, and bacterial leaf spot in tomato to name a few (Sarkar *et. al*., 2018). Very recently, the state government of West Bengal had established fair price outlet for medicines where amikacin (250 / 500 mg vial injection) is included as important medicine under mandatory list (document entitled " Establishment of fair price outlet for medicines, consumables and implants at selected government hospitals through public private partnerships (PPP); http://www.wbhealth.gov.in> upload files > PPP > ppp_02) and sold from district and sadar hospitals in large quantities to treat diseases caused by gram-negative pathogens; unprecedented use of amikacin in hospitals and community may have been the reason for the widespread dissemination and enrichment of *aac* (6') Ib gene in integrons.

Two Karala river MBL positive *Proteus* spp., bearing class 1 integron having two gene cassettes, *aac* (6')-I-*aad*A2, and *aad*A2-*aad*A1 respectively and it also to bear $bla_{\text{NDM-1}}$ MBL gene. In a study carried out in the USA, two *P. mirabilis* isolates was found to contain $bla_{\text{IMP-27}}$ gene as the integral part of class 1 integron (Dixon *et. al*., 2016). In a hospital isolate, *P. mirabilis*, was reported to bear $bla_{\text{VIM-1}}$ and *aad*A2 cassettes in class 1 integron (Qin *et. al*., 2015).

The whole genome sequencing in the current study was done to explore the resistance pattern (intrinsic and adaptive antibiotic resistance genes) of Mahananda River MBL positive isolates *Pseudomonas* sp. MR 02 at molecular level. The resulting whole genome was to allow comparison between MR 02 genome and to other previously sequenced *Pseudomonas* species and also to help in the comparative analysis between phenotype and genotype characters (Figure 4.10).

The choice of *Pseudomonas* sp. MR 02 isolates for whole genome sequencing was based on antibiotic susceptibility pattern. It showed resistance against all antibiotics

categories which is defined for pan-drug resistance (PDR) strain by CLSI and EUCAST (antibiotic list for *Pseudomonas aeruginosa* described by Magiorakos *et. al*., 2012). In this study MR 02 isolates are genotypically and phenotypically characterised a PDR strain.

Two different bioinformatic tools, CARD and BLAST were applied in the antibiotic resistance gene analysis from whole genome sequence of *Pseudomonas* sp. MR 02 isolates. In draft genome of MR 02, 98 genes were identified as antibiotics resistance related genes and among this a total 64 genes have been predicted as antibiotic resistance efflux pumps. They cover all the five known efflux pump families of bacteria namely MFS, MATE, SMR, ABC and RND. Altogether eleven RND efflux pumps have been predicted in MR 02. One of the most relevant efflux pump families in the clinical context is the RND family, which has been characterized best in Gram-negative bacteria (Fernandez and Hancock, 2012). In general, RND efflux pumps consist of three components but in MR 02 single set of four component RND efflux pump TriABC-OpmH and MuxABC-OpmB has been found, same is the case with *P. aeruginosa* (Mima *et. al*., 2007 & 2009). One set of ABC type efflux pump Mac AB has been predicted in MR 02 and they show homology with *E. coli* Mac AB. Mac AB pump has involved in macrolide-specific resistance in *E. coli* (Kobayashi *et. al*., 2001). RND efflux pumps like MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexVW-OprM; MFS efflux pumps like TetG, EmrB, EmrAB and MdtA; MATE efflux pumps like NorM and SMR efflux pumps like emrE have identified from draft genome of MR 02.

Presence of mutated genes like *gyrA* and *parC* along with the abovementioned efflux channels might have played a role in development of resistance to fluoroquinolones in MR 02 (Yoshida *et. al*., 1990; Piddock *et. al*., 1999). 14 OprD porin channel genes were predicted. OprD is the major channel for entry of carbapenems. In *Pseudomonas aeruginosa* the most common mechanism of resistance to the cabapenems is loss or alteration of the outer membrane porin protein OprD (Atsuyuki *et. al*., 2009). Out of 14 porin channels annotated in MR 02 of which only 5 channels exhibited below 50% homology with *Pseudomonas aeruginosa* PA01 porin channels.

The resistance phenotype against the β-lactam antibiotics was corroborated with the genomic presence of three different β-lactamases, PME-1, NDM-1 and AMPC, of which PME-1 and NDM-1 exhibited 100% similarity with the reported sequences and only 52%

similarity with AMPC reported from *Pseudomonas aeruginosa* PA01 (PDC-1) sequence. PME-1 β-lactamase enzyme was first time detected in *Pseudomonas aeruginosa*, which is isolated from clinic sample. It was grouped in ambler class A extended-spectrum β-lactamase (ESBL) and conferred resistance to cephalosporin and aztreonam antibiotics but not to carbapenems (Tian *et. al*., 2011). Presence of NDM-1 in *Pseudomonas aeruginosa* was reported for the first time from Serbia (Jovcic *et. al*., 2011). AmpC enzymes are not carbapenemases, they posses however a low potential for carbapenem hydrolysis. But the combined effect of AmpC enzyme and the efflux pumps lead to carbapenem resistance in *P. aeruginosa* (Livermore, 1995 & 2002).

Active efflux of antibiotics is a major way by which bacteria can become multiple-antibiotic-resistant. Phenylalanine-arginine-β naphthylamide (PAβN) is a well-studied efflux pump inhibitor. The MICs of chloramphenicol and tetracycline were reduced to 2.0 % of their values shown in absence of PAβN, showing that the susceptibility changes were entirely due to efflux inhibition (Figure 4.). Similar significant reduction in MIC values for fluoroquinolone antibiotics, ciprofloxacin and levofloxacin, was observed in presence of PAβN. In *P. aeruginosa*, an extensive range of antibiotics groups (except polymixin B and imipenem) comprising of quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, most penicillins except carbenicillin and subbenicillin, most cephems except cefsulodin and ceftazidime, meropenem, and S-4661, are driven out of the cell by efflux systems (Masuda *et. al*., 2000; Lamers *et. al*., 2013). Besides Mex AB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM, there are several additional chromosomally encoded efflux pumps like MexHI-OpmD, MexVW-OprM, the NorM ortholog, and mutations of certain genes (*gyrA*, *gyrB*, *parC*, and *parE*) were identified responsible for development of resistance to quinolone group of antibiotics in *Pseudomonas aeruginosa* (Yoshida *et. al*., 1990; Piddock *et. al*., 1999). To explain the molecular basis of MR 02's efflux-dependent resistance against chloramphenicol, tetracycline, ciprofloxacin, and levofloxacin, we were able to assign 8, 11, 5, and 5 efflux-system genes/gene-clusters annotated from its whole-genome-sequence. In addition to efflux genes, two mutated genes, *gyr*A and *par*C, were also assigned for imparting resistance to ciprofloxacin or levofloxacin.

At the end of this study, relevant role of *bla*$_{NDM-1}$ genes in PDR strain *Pseudomonas* sp. MR 02 was described for ampicillin catabolism. While determining the MIC of different antibiotics, very high MIC values were obtained against the ampicillin (10000 mg/L). This observation led us to re-think this issue from the bacterial perspective, with cleaved β-lactam ring of the antibiotic, the inactivated molecule is nontoxic and can be exploited as carbon and nitrogen sources, allowing the bacteria to grow in elevated concentration of the drug. The role of NDM-1 in ampicillin catabolism was established because the growth of MR 02 in the same ampicillin containing minimal media was inhibited, when we added 0.4 mM EDTA. NDM-1 is known to be more susceptible to EDTA than other metallo-β-lactamase (Li *et. al*., 2013). In this study, 0.4 mM EDTA was used to completely inhibit NDM-1 activity; which in turn did not allow MR 02 to grow on ampicillin. To rule out any untoward effect of 0.4 mM EDTA, uninhibited growth of MR 02 was demonstrated in minimal medium containing glucose and NH_4Cl (Figure A5.1).

Long back, in 1977, a *Pseudomonas fluorescence* strain with β-lactamase activity was reported to have the ability to use benzylpenicillin as a sole source of both carbon and nitrogen (Beckman & Lessie, 1979). Later in 1994, *Phormidium valderianum* BDU30501 strain was shown to utilize ampicillin as nitrogen source via a probable mechanism of splitting of 6-aminopenicillanic acid into cysteine and valine (Bosnic *et. al*., 1994). Taking the cue from a very recent report by Croft *et. al*., 2018 that the non-β-lactam region of pencillin, phenylacetamide is capable to support growth of bacteria via phenyl acetic acid catabolon, we hypothesized on the basis of earlier report ampicillin binds to the two zinc ions and various critical residues present in the active site of NDM-1 through number of coordination bonds and hydrogen bonds that might render the host bacterium an opportunity to utilize the ampicillin as sole source of carbon, nitrogen, and energy. We were successful to demonstrate growth of MR 02 on ampicillin not only as sole carbon source but both sources of carbon and nitrogen. Despite having a 6-aminopenicillanic acid core containing nitrogen in all β-lactam antibiotics, ampicillin possesses a side chain with an additional amino group. From the results, it was revealed that ampicillin is also being used by MR 02 as nitrogen source.

Moreover in support of the hypothesis, we assayed growth of the recombinant *E. coli* DH5α: pHSG398: *bla*$_{NDM-1}$ strain using ampicillin (100 mg/L) as the only carbon

source, we found that expression of bla_{NDM-1} was sufficient to confer a significant role in ampicillin catabolism. These results confirmed the validity of our conjecture as well as the role of NDM-1 in ampicillin catabolism.

The comparative transcriptome analysis was performed between minimal media with glucose as carbon source and minimal media with ampicillin as carbon source grown MR 02 cells. After RNA sequencing and data analysis, 3707 DEGs were identified between two samples. It was revealed that there has been alteration of the expression of metabolic genes to meet growth requirements in mineral medium containing ampicillin as sole carbon and energy source. MR 02 cells might have employed gluconeogenesis pathway to synthesize glucose-6-phosphate to make various cell macromolecules (as glucose was not available from the cell surroundings). The expression of *pck*, *gap*N, *pgm*, *adh*C, *gpm*I, and *tpi*A were higher along with significant upregulation of *ald*B (coding for aldehyde dehydrogenase) and *exa*A (coding for PQQ dependent dehydrogenase) genes of the gluconeogenesis pathway (Table A5.1). Besides gluconeogenesis pathway genes, three genes of the TCA cycle coding for NADP-dependent isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase E1 component (*suc*A), succinate dehydrogenase flavoprotein subunit (*sdk*A, *frd*A); and one gene coding for D-glycerate dehydrogenase (phosphor-gluconate 2-dehydrogenas) of pentose phosphate pathway were found to be significantly upregulated, suggestive of regulation of genes related to growth on ampicillin as sole carbon and energy source. On the other hand, two genes coding for NADPH dehydrogenase (quinone) of ubiquinone and terpenoid-quinone biosynthesis pathway was significantly downregulated (Table A5.2). MR 02 cells grown in both conditions (glucose or ampicillin) have expressed genes for PQQ-dependent dehydrogenases to use a PQQ-dependent ethanol oxidation system with exception of significant upregulation of two exaA genes (coding for PQQ-dependent dehydrogenase, methanol/ethanol family), and one exaC gene (coding for aldehyde dehydrogenase) in ampicillin-grown cells. Interestingly, significant differences in expression of genes coding for proteins essential for the biosynthesis of the cofactor PQQ, organised in an operon *pqq*ABCDE and *pqq*F (expressed singly) were not observed in ampicillin or glucose-grown cells. Four genes coding for different aminotransferase enzymes (02 for cysteine and methionine metabolism; 01 each for thiamine and propanoate metabolism respectively), one gene

coding for FAD-binding oxidoreductase (of the arginine and D-ornithine metabolism), two genes coding for asparaginase (of the alanine, aspartate and glutamate metabolism), one gene coding for acyloxyacyl hydrolase (of the lipopolysaccharide biosynthesis), and *hmg*L gene for hydroxymethylglutaryl-CoA lyase (for butanoate/ valine, leucine and isoleucine degradation/geraniol degradation pathway) were significantly upregulated; while two genes coding for dihydrofolate reductase (for folate biosynthesis) and 2-hydroxy-3-oxopropionate reductase (for glyoxylate and dicarboxylate metabolism) respectively were significantly downregulated in ampicillin-grown cells (Table A5.2). Two genes coding for peptidases were found to be significantly downregulated (M23 family metallopeptidase, LD-carboxypeptidase, and aminopeptidase) and upregulated (ATP –dependent Lon protease and S24 family peptidase) respectively in ampicillin grown cells.

Overall it was observed that, for ampicillin catabolism three components are required and according to my hypothesis, when any bacterial species carries all these three components they are able to grow in ampicillin by consuming ampicillin as carbon and nitrogen source. Again to validate this hypothesis, we selected four different MBL producing strains; *bla*$_{\text{NDM-1}}$ positive *Proteus* sp. KR01, *bla*$_{\text{NDM-7}}$ positive *Escherichia* sp. KR23, *bla*$_{\text{VIM-2}}$ positive *Pseudomonas mendocina* KR25 and *bla*$_{\text{NDM-1}}$ *Acinetobacter* sp. MR40 for ampicillin catabolism, which are isolated from both rivers. Among these four strains only two strains KR23 and MR40 were able to grow in ampicillin containing minimal media. When KR01 and KR25 bacterial strains were grown in minimal media with ampicillin as carbon source no significant growth was observed and this result also validated our hypothesis. On the basis of whole genome analysis of *Proteus mirabilis* phylogenetically nearest species of KR01, and we found that they did not have any PAA pathway related genes but in *Acinetobacter variabilis* nearest species of MR40 have other two component amidase and PAA genes which is required for ampicillin catabolism.

Summary

While a few reports give proof of the event of NDM-1-positive bacteria in tertiary healthcare centers of West Bengal, India, a finding of apparently more noteworthy for the general well-being has been given in the present study with water tests from two rivers bisecting two crowded towns, Siliguri and Jalpaiguri separately. No significant variation was detected in imipenem resistant bacteria counts between both river water samples. All the 15 MBL positive strains were isolated from the mid steam water sample. The most abundant and most common MBL in both river water samples were NDM (94%). The investigation has demonstrated that the issue of NDM-1 was not kept to clinical pathogenic strains, limited within the hospital environment but also has stretched its residence in the environmental bacteria. The spread of NDM has been perplexing the study of disease transmission, including the spread of an assortment of types of NDM-positive bacteria and the interstrain exchanges between species and between genera via the transmission of various mobile genetic platforms like plasmids and integrons containing bla_{NDM}, with the last instrument having assumed a progressively noticeable impact to date. Nearly 50% (8/15) MBL positive strains are carrying aminoglycosides resistance gene cassettes. The NDM gene was not evolved only for β-lactam antibiotic inactivation rather it also catabolized ampicillin and it provides carbon and nitrogen from ampicillin molecules to bacterial cell for growth and other energy sources. The spread of NDM shows that antimicrobial resistance is a general medical issue that rises above national fringes and will require worldwide collaboration between experts on the off chance that it is to be controlled.