# Chapter 4

## Whole-genome sequencing of a model *Pseudomonas* sp. MR 02 bearing *bla*$_{\text{NDM-1}}$ gene

## 4.1 Introduction

The infection which is caused by antibiotic-resistant bacteria constitutes one of the most serious threats to human health. If we leave it uncontrolled, even small surgery can become a major risk procedure (Davies, 2011). Controlling of antibiotic resistance bacterial infection is becoming more complex than previous to, because there is a huge difference between antibiotic development and antibiotic resistance character developed by microbes and this difference can be controlled by using rapid and improved diagnostics techniques (WHO, 2012; Livermore & Wain, 2013; Spellberg, 2013). As a result of technological development in recent times, bacterial whole genome sequencing has emerged as a tool in clinical microbiology and research fields that can reduce this gap. Whole genome sequencing of bacteria has emerged as a cost-effective and convenient technique for identification of bacterial behaviours in single assays. Although in traditional method separate technique is used for species identification and virulence and drug resistance character identification. A major advantage of whole genome sequencing is to yield all of the available genetic information in single steps of bacterial culture (Loman, 2012; Loman, 2013).

After the introduction of next-generation sequencing (NGS) in 2005 was enabled to reduce the sequencing cost and facilitated the performance of sequencing in all types of laboratories (Barbosa *et al*., 2014). Several NGS sequencing technology available at the current time, but the general steps remain the same, and it includes sample preparation, DNA sequencing, sequence assembly and bioinformatics analysis (Dark, 2013). These NGS techniques include the Illumina Miseq and Hiseq (both are accurate with low error rates), SOLiD (Sequencing by Oligo Ligation Detection) developed by Life technologies and in several countries, such as USA, United Kingdom and Germany Whole-genome sequencing technique is also being used or in a trial phase in the medical microbiology field, because this technique allows rapid detection of pathogenic character from infected human and to direct appropriate treatment (Pareek *et. al*., 2014; Pecora *et. al*., 2015; Zankari *et. al*., 2013; Koser *et. al*., 2014; Mellmann *et. al*., 2017). A major advantage of whole-genome sequencing is to yield all of the available genetic information in single steps of bacterial culture and directly to identify a microorganism and also establish genotypic to phenotypic relationships (Torok *et. al*., 2012).

The first complete bacterial genome to be sequenced was that of *Haemophilus influenzae* in 1995 and the *Pseudomonas aeruginosa* PA01 was the fifth bacterial strain whose complete whole-genome reported in 2000 (Fleischmann *et. al*., 1995; Stover *et. al*., 2000). *Pseudomonas aeruginosa* ubiquitous in nature, and is present in soil and water as well as on fruit, vegetables and flowers (Kiska & Gilligan, 2003) and it can also be isolated from human, animal and clinical settings (Lederberg *et. al*., 2000). *Pseudomonas aeruginosa* is an opportunistic human pathogen and it is responsible for 18% nosocomial infection in human. They were first isolated and identified in 1850 by Migula (Lederberg *et. al*., 2000). Genus *Pseudomonas* is represented by species that occupy a wide range of niches owing to metabolic and physiological diversity (Kiska & Gilligan, 2003). *Pseudomonas aeruginosa* strain contains approximately 5 to 7 Mbp genome size, and it contains significant numbers of regulatory protein-encoding genes. This indicates that *P. aeruginosa* is capable of responding to various environmental stresses (Moradali *et. al*., 2017). Additionally *P. aeruginosa* has intrinsic multidrug resistance properties due to the presence of three or four component like RND efflux pumps, porin channel and AMPC β-lactamase that physically sequester incoming antibiotics and hydrolyzed incoming β-lactam antibiotics (Aeschlimann, 2012; Jacoby, 2009). Moreover, biofilms act as a protective layer for drug penetration (Stewart, 2000). Genetically it can also receive the antibiotic resistance gene from other species by a horizontal transfer mechanism (Davies and Davies, 2010).

In the current study, NDM-1 producing *Pseudomonas* sp. MR 02 was the only strain found in Mahananda River, which is isolated in two different sampling dates and it has also high MIC value against different antibiotics. However, when applying genotypic screening to detect carbapenem resistance gene, PCR assays to detect only one MBL which was NDM-1, but in the metallo-β-lactamases activity test of MR 02 strain, the MIC value of cephalosporin antibiotics in the presence of EDTA (0.4 mM) was recorded higher than the resistance MIC value (CLSI guideline) and these results indicate that other genes are also playing a role in cephalosporin resistance. NDM-1 metallo-β-lactamases are responsible for hydrolyzing β-lactam antibiotic groups but *Pseudomonas* sp. MR 02 Mahananda river water isolates showing resistance in lactams group antibiotic as well as in other antibiotic groups like aminoglycosides, chloramphenicol, tetracycline, trimethoprim

and fusidic acid. This result highlights the limitations of genotypic and phenotypic test for detecting and allowing characterization of divergent functional genes. Hence in this chapter, NGS to the analysis of multiple drug resistance *Pseudomonas* sp. MR 02 isolates especially. The objective of this chapter was to describe the diversity and distribution of resistance mechanism and also corroborate genotypic-to-phenotypic relationships in MR 02 isolates.

## 4.2 Materials and Methods

### 4.2.1 Isolation of genomic DNA from *Pseudomonas* sp. MR 02

*Pseudomonas* sp. MR 02 genomic DNA was isolated by using phenol: chloroform isolation method described by Furlong *et. al*., (2002) with modifications. The quantity of genomic DNA was measured using a NanoDrop spectrophotometer, while its quality was assessed via agarose gel (0.8%) electrophoresis.

### 4.2.2 Sequencing of *Pseudomonas* sp. MR 02 genome

The genome of strain MR02 was sequenced by the NGS Laboratory (Eurofins Genomics, India) using de novo sequencing by Illumina NextSeq 500 platform and methods described below.

#### 4.2.2.1 Preparation of 2×150 NextSeq 500 shotgun libraries

The paired-end sequencing libraries were geared up using illumina TruSeq Nano DNA Library Prep Kit. Briefly, approximately 200 ng genomic DNA of MR 02 was fragmented by covaris M220 to generate fragment distribution of 400bp. Covaris shearing makes dsDNA fragments with 3 or 5 overhangs. The fragments were then subjected to end-repair. The 3 to 5 exonuclease activity of this mix removes the 3 overhangs and the 5 to 3 polymerase activity fills in the 5 overhangs followed by adapter ligation to the fragments. Ligated and end-repaired products were size selected by AMPure XP beads. The size selected products were PCR amplified with index primer and indexing adapters were ligated to the ends of the DNA fragments, preparing them for hybridization onto a flow cell. The library was analyzed in a 4200 Tape Station system (Agilent Technologies).

#### 4.2.2.2 Cluster generation and sequencing

After quantity and quality check of library Paired-end Illumina libraries were loaded onto NextSeq 500 for cluster generation and sequencing. Paired-end sequencing

allows the DNA template fragments to be sequenced in both directions on NextSeq500. After both template fragments sequencing, 1.1 GB adapter trimming data of MR 02 was generated, which was required for the genome to be used in a taxonomic purpose (Chun *et. al.,* 2018).

#### 4.2.2.3 Genome annotation and analysis

The high- quality filtered reads were assembled into scaffold using SPAdes genome assembler (Bankevich, 2012). Prokka v-1.12 (Seemann, 2014) was used to predict the genes from final complete assembled scaffolds. BLASTx (NCBI) programme were used for functional annotation of the MR 02 gene, and Blast2GO platform were used for MR 02 gene ontology annotations (Jones *et. al*., 2008).

### 4.2.3 Identification of antibiotic resistance genes in *Pseudomonas* sp. MR 02

AMR (antimicrobial resistance) genes were identified in draft genome of MR 02 using publicly available comprehensive antibiotic resistance databases (CARD) (Jia *et. al*., 2017). Resistance determinants were identified proviso they conform the criteria of = 85% amino acid identity and =50% sequence length identity to known resistance proteins. Sequences showing = 100% identity and/or sequence length were analyzed by additional BLAST analysis to identify the appropriate resistance genes. The results obtained were then again validated by BLAST analysis against information in the COG, NCBI, CDD and Swiss-Prot databases; manual curation was done for all blast results as per consistency of annotations between different databases. For analysis of chromosomal structural gene mutations, *gyrA* and *parC*, genes, were analyzed for quinolone resistance-determining region (QRDR) mutations, with alignment by ClustalW in Mega version 7 (Park *et. al*., 2011).

### 4.2.4 Phylogenetic and comparative genomic analysis of *Pseudomonas* sp. MR 02 with reference genome of *Pseudomonas* spp.

*Pseudomonas* sp. MR 02 were subjected to phylogenetic analysis using four different method viz. 16S rRNA based phylogenetic analysis (chapter 2), bcg tree based phylogenetic analysis, Average Nucleotide Identity (ANI) and *In silico* DNA-DNA hybridization (*i*DDH).

For comparative genome analysis, the complete genome sequence of strain MR02 along with that of twenty *Pseudomonas* type strains were obtained and used as references

for building of phylogenetic tree based on core genes. A phylogenetic tree based on bacterial core genome was constructed by the bcgTree pipeline (Vesth *et. al*., 2013; Ankenbrand & Keller, 2016). The pipeline automatically extracts 107 essential single-copy core genes, found in a majority of bacteria, using hidden Markov models and uses them to reconstruct a phylogenetic tree based on partitioned maximum-likelihood analysis utilizing RAxML program (Stamatakis, 2014).

Average Nucleotide Identity (ANI) was calculated by the recently released OrthoANI program (Lee *et. al*., 2016). The OrthoANI provides a measure of genomic relatedness correlated with the original ANI and both of these algorithms share the same species demarcation cut-off at ≤ 96%. In addition, OrthoANI revolves around the problem of reciprocal inconsistency of the original ANI algorithm. ANI value to define a new species is ≤ 96.

*In silico* DNA-DNA hybridization (*i*DDH) was estimated by using the Genome-to Genome Distance Calculator (GGDC) version 2.1 Web server (http://ggdc.dsmz.de/ggdc.php) (Auch *et. al*., 2010). Since many of the genomes are incomplete draft genomes, therefore Formula 2 was used for the analysis. Formula 2 calculates *i*DDH estimates independent of genome lengths and is recommended by the authors of GGDC for use with any incomplete/draft genomes. The point estimate plus the 95% model-based confidence intervals were used for analysis. DDH value to define a new species is ≤ 70%.

The contigs sequence of *Pseudomonas* sp. MR 02 was aligned to the complete sequence of *P. putida* NDRC14164 genome using Muscle (Kurtz *et. al*., 2004) for making genome orientation. Four available whole genome sequences of the phylogenetically nearest species *P. plecoglossicida* DSM15088, *P. monteilii* NBRC103158, *P. putida* NBRC14164 and *P. aeruginosa* PA01 were used for the comparative genomic analysis with MR 02. For visualization of circular genome comparison, the BLASTN-based graphical map was generated by BLAST Ring Image Generator (BRIG) 0.95 (Alikhan *et. al*., 2011).

### 4.2.5 Correlation of phenotypic and genotypic characters of MR 02

Two different types of phenotypic experiments used for antimicrobial susceptibility testing one for efflux pump activity detection and another one for *bla*$_{NDM-1}$ activity (Chapter 1 in section 1.2.9). Each interpretation of antibiotics resistant was compared with the presence of known corresponding resistance genes and/or structural gene mutations.

### 4.2.5.1 Determination of efflux pump activity in contributing antibiotic-resistance in MR 02

The efflux pumps activity in MR 02 isolates was confirmed by the micro-broth-dilution method, in Mueller Hinton broth. It is based upon the comparative MIC values of antibiotics were also determined in the presence of Phe-Arg-β-naphthylamide (30 μg/ml) and absence of Phe-Arg-β-naphthylamide. The antibiotics were used as phenotypic markers of the RND efflux pumps of interest shown in Table 4.1.

**Table 4.1: Examples of RND efflux systems involved in the antibiotic resistance in *Pseudomonas aeruginosa* and *Pseudomonas* species**

| Efflux pump types | Antibiotic resistance to | References |
|---|---|---|
| MexAB-OprM | β-lactams, Azithromycin, Chloramphenicol, Macrolides, Tetracycline, Trimethoprim, Imipenem , Meropenem | Li *et. al*., 1995; Poole *et. al*., 1993; Poole *et. al*., 1996 (a) |
| MexCD-OprJ | Cephalosporins, Chloramphenicol, Fluoroquinolones, Tetracycline, Imipenem | Poole *et. al*., 1996 (b) |
| MexEF- OprM | Chloramphenicol, Fluoroquinolones, Imipenem, Meropenem | Kohler *et. al*., 1997 |
| MexJK-OprJ | Aminoglycosides, Ciprofloxacin, Erythromycin, Tetracycline | Chuanchuen *et. al*., 2002 |
| MexVW- OprM | Chloramphenicol, Fluoroquinolones, Tetracycline | Li *et. al*., 2003 |
| MuxABC-OprM | Novobiocin | Mima *et. al*., 2009 |
| TriABC-OprH | Triclosan | Mima *et. al*., 2007 |

MICs were determined by broth microdilution in the absence or presence of a broad-spectrum inhibitor of RND pumps, namely Phe-Arg-β-naphthylamide (PAβN), also

known as MC-207,110 at a concentration of 50 μg/ml, which fully restored the susceptibility of reference strains to the reporter antibiotics but did not impair the bacterial growth **(**Lomovskaya *et. al*., 2001**)**. The test tube was prepared with Mueller Hinton broth, antibiotics (different concentration), Phe-Arg-β-naphthylamide (fixed concentration) and MBL producing bacterial cells and test tube incubated for 24 hours at 37°C. The inoculums used per experiment was $10^4$ to $10^5$ cells/ml. Efflux pump activity was determined as the lowest concentration of antibiotics with fixed concentration of efflux pump inhibitor that completely inhibited growths of the MBLs producing bacterial strain in the test tube, and bacterial growth was detected by turbidity measurements.

To detect minimal changes, however, arithmetic dilutions were occasionally used for all the antibiotics used in the study (10 μg/ml increments in the 10–2000 μg/ml range).

### 4.2.5 Nucleotide sequence accession numbers

The whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number PESJ00000000.

## 4.3 Results and Discussion

### 4.3.1 Isolation, qualitative and quantitative analysis of *Pseudomonas* sp. MR 02 genomic DNA

Qualitative analysis of the strain MR 02 genomic DNA was resolved on 0.7% agarose gel at 100 V for approximately 60 min or till sample reached 3/4$^{th}$ of the gel (Appendix A 4.1) and 1 μl of the sample was loaded in nanodrop for quantitative analysis. The quantification of genomic DNA is an important step before starting the genome sequencing. Concentration and A260/280 ratio of genomic DNA of strain MR 02 was 425 and 1.76 respectively. For whole genomic sequencing, the DNA sample preparation is very important as the optimal quantity and quality of genomic DNA is crucial. The high and low concentrations of genomic DNA will affect the sequencing quality; if the DNA concentration is high these will produce overlapping sequencing clusters. On the other hand, if the genomic DNA is low than the optimal amount needed, then it will give the clusters with low density (Buehler *et. al*., 2010)

### 4.3.2 Whole genome sequencing, genome annotation and analysis of *Pseudomonas* sp. MR 02

The QC passed sample was used to prepare paired end library using Illumina TrueSeq Nano DNA library preparation kit generating a fragment with mean length of 434bp of strain MR 02. The libraries were sequenced on NextSeq 500 using 2×150bp. After sequencing adapter was removed by trimming (trimmomatic v0.35) and obtain high quality data (1.1 GB with 1,074,251,227 bases).

SPAdes genome assembler tool used for de novo assembly of MR 02 high quality read. Arranged genome in scaffolds and detailed assembly statistics were mentioned in the Table 4.2.

**Table 4.2: Draft genome assembly statistics of *Pseudomonas* sp. MR 02**

| Scaffolds | Total size of assembly (bp) | Average size of scaffolds (bp) | N50 (bp) | Max size of scaffolds (bp) | Min size of scaffolds (bp) |
|---|---|---|---|---|---|
| 90 | 5,946,931 | 66,077 | 144,752 | 431,092 | 520 |

Protein coding sequencing were predicted from the assembled scaffolds using Prokka v-1.12 with default parameters. Statistics of total number of genes, average gene length, maximum and minimum length of the strain MR 02 was 5,661, 923, 12,954, and 59 respectively.

Functional annotation of the MR 02 genes was performed using BLASTx program and it has a part of ncbi blast 2.3.0+ search application. BLASTx find the homologous sequences for the genes against NR (non redundant protein database) from NCBI, Swiss-prot database, KEGG (Kyoto Encyclopedia of Genes and Genomes), COG (Clusters of Orthologous Groups) and InterPro database with an E-value $<10^{-5}$. After blast analysis MR 02 draft genome consist of 5,530 genes, out of which 5,493 protein coding genes showing blast hits and 37 protein coding genes showing without blast hit. Top blast hit species distribution of MR 02 showing in Figure 4.1.

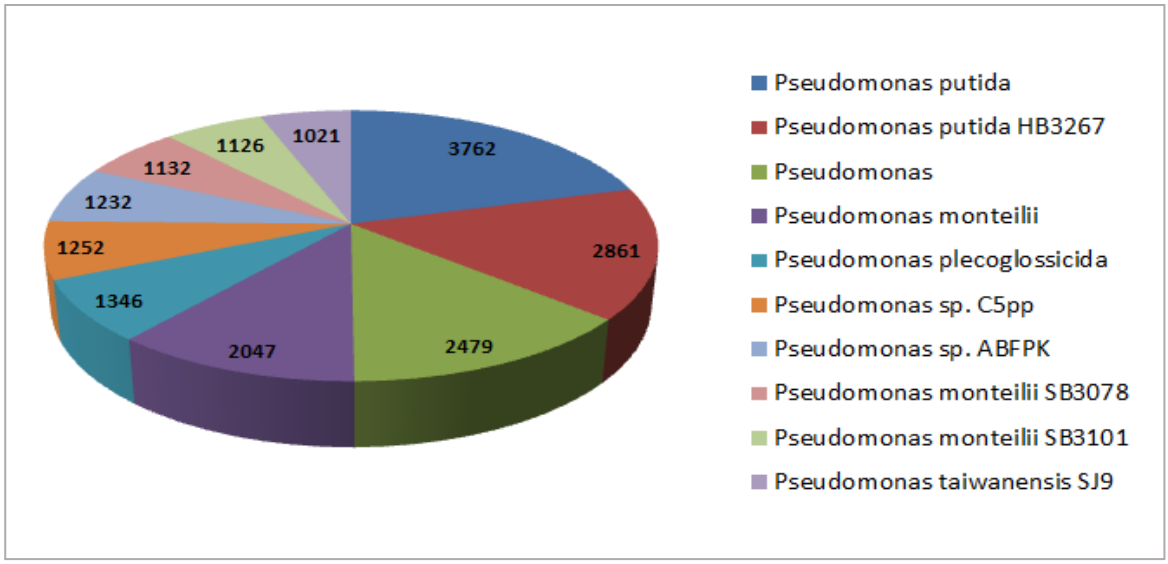


**Figure 4.1: Top blast hit species distribution of *Pseudomonas* sp. MR 02**

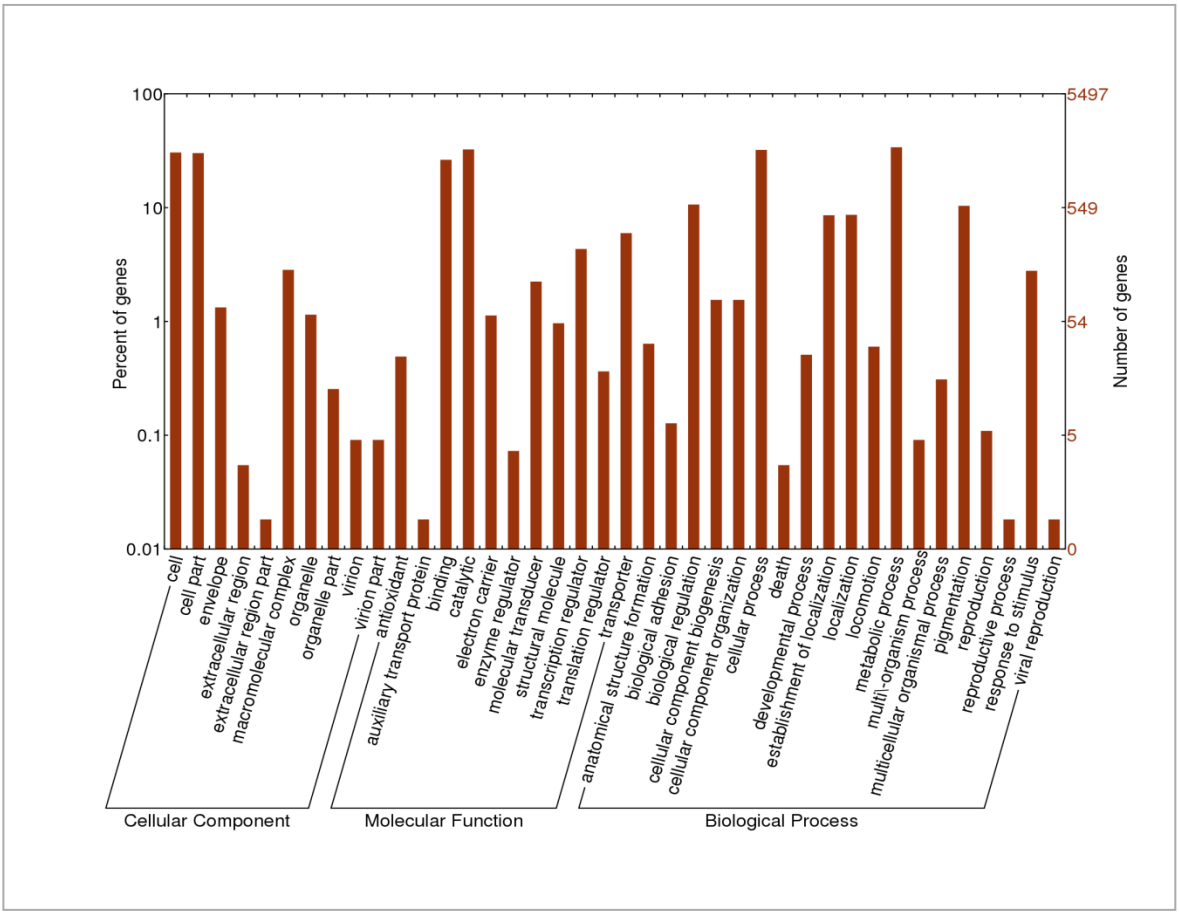


**Figure 4.2: WEGO plots of the *Pseudomonas* sp. MR 02**

BLASTx results accession IDs (functionally annotated genes) were directly searched in the gene products table of gene ontology database. Gene Ontology mapping provided function of the genes and their product properties. On the basis of genes product properties Blast2Go platform MR 02 genes are grouped into three main domains: Biological processes (2,593), Cellular component (1,687), and molecular function (2,695). Graphical-representation of MR 02 draft genome ontology in figure 4.2. Basic feature of MR 02 draft genome are summarized in table 4.3. Most of the predicted ORFs have the high G+C contents (67.6%).

**Table 4.3: Genome features of *Pseudomonas* sp. MR 02**

| **General feature** | | | |
|---|---|---|---|
| **Genome size** | 5,946,931 | | |
| **G+C content** | 62.6% | | |
| **Coding regions** | | | |
| **Genes (Total)** | 5,799 | | |
| **CDS (Total)** | 5,732 (98.84%) | | |
| **Genes (coding)** | 5,503 (94.89%) | | |
| **CDS (coding)** | 5,503 (94.89%) | | |
| **Genes (RNA)** | 67 | | |
| **Types of rRNA** | 16S rRNA | 23S rRNA | 5S rRNA |
| **Number of rRNA** | 1 | 1 | 2 |
| **tRNA** | 59 | | |
| **Non-classical RNA** | 4 | | |
| **Pseudogenes (total)** | 229 | | |

### 4.3.3 Identification of antibiotic resistance in *Pseudomonas* sp. MR 02

Protein sequences of MR 02 draft genome were used for searches against CARD, and NCBI protein smart blast. After comparative analyses of the draft genome of *Pseudomonas* sp. MR 02, we identified a total of 98 genes associated with antibiotic resistance. Total 98 (1.78%) antibiotics resistance genes were compared with *Pseudomonas aeruginosa* PA01 (Table 4.4). 34 (0.67% of total ORFs) intrinsic antibiotics resistance genes are present in *P. aeruginosa* PA01 genome. On the basis of these chromosomally encoded antibiotics resistance gene *P. aeruginosa* is naturally resistance

against β-lactams, some aminoglycosides, nalidixic acid and trimethoprim antibiotics (Wroblewska, 2006; Stover *et. al.*, 2000). Total 8 genes including metallo-β-lactamases *bla*$_{NDM-1}$ gene, that is absent in PA01.

**Table 4.4: Total antibiotics predicted genes in *Pseudomonas* sp. MR 02**

| MR 02 | | | | Query Cover with highest homology one | PA01 | | |
|---|---|---|---|---|---|---|---|
| **Annotated Protein of MR 02** | **Name of the Genes** | **Accession No. of annotated protein** | **Number of amino acid Predicted** | | **% identity with PA01** | **Accession No.** | **Number of amino acid** |
| **RND** | *oprN* | PJI75874·1 | 509 | 93% | 64% | NP252583 | 496 |
| **MFS** | *emrA* | PJI75872·1 | 286 | 98% | 73% | NP252581 | 302 |
| **MFS** | *emrB* | PJI75869·1 | 513 | 100% | 60% | NP251525 | 530 |
| **MFS** | *emrA* | PJI75868·1 | 353 | 86% | 66% | NP251526 | 354 |
| **RND** | *oprM/opmB* | PJI75867·1 | 483 | 96% | 56% | NP251527 | 479 |
| **PBP2** | | PJI75810·1 | 581 | 99% | 79% | NP253108 | 579 |
| **MFS** | *mdfA* | PJI75788·1 | 414 | 68% | 31% | NP252825 | 402 |
| **RND** | *mexW/mexI* | PJI75774·1 | 1014 | 99% | 82% | NP253065 | 1018 |
| **RND** | *mexH* | PJI75773·1 | 382 | 97% | 60% | NP253064 | 376 |
| **Transcriptional regulator** | *mexT* | PJI7571·1 | 307 | 99% | 66% | NP248881 | 305 |
| **Porin** | *oprD* | PJI75717·1 | 418 | 96% | 72% | NP248931 | 421 |
| **RND** | *oprM* | PJI75716·1 | 485 | 96% | 70% | NP249118 | 485 |
| **RND** | *mexB* | PJI75715·1 | 1050 | 99% | 78% | NP249117 | 1045 |
| **RND** | *mexA* | PJI75714·1 | 384 | 99% | 67% | NP249116 | 383 |
| **MFS** | *emrB* | PJI75712·1 | 483 | 98% | 66% | NP248937 | 501 |
| **Porin** | *oprD* | PJI75663·1 | 429 | 93% | 65% | NP249446 | 427 |
| **MATE** | *norm* | PJI75496·1 | 462 | 98% | 61% | NP253981 | 488 |
| **Porin** | *oprD* | PJI75483·1 | 420 | 93% | 66% | NP251390 | 435 |
| **RND** | *triA* | PJI75410·1 | 362 | 95% | 57% | NP248846 | 383 |
| **RND** | *triB* | PJI75409·1 | 455 | 98% | 60% | NP248847 | 356 |
| **RND** | *triC* | PJI75408·1 | 1018 | 99% | 74% | NP248848 | 1015 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Dihydrofolatereductase** | *dfrA* | PJI75361·1 | 171 | 96% | 66% | NP249041 | 168 |
| **Porin** | *oprD* | PJI75048·1 | 429 | 100% | 63% | NP252868 | 431 |
| **RND** | *opmH* | PJI74863·1 | 452 | 89% | 24% | NP250566 | 425 |
| **RND** | *mexK* | PJI74743·1 | 1021 | 99% | 83% | NP252366 | 1025 |
| **RND** | *mexJ* | PJI74742·1 | 366 | 100% | 70% | NP252367 | 367 |
| **DNA topoisomerase IV subunit A** | *parC* | PJI74590·1 | 752 | 99% | 87% | NP253651 | 754 |
| **RND** | *opmH* | PJI74580·1 | 478 | 100% | 73% | NP253661 | 482 |
| **SMR** | *emrE* | PJI74577·1 | 110 | 100 | 64% | NP253677 | 110 |
| **RND** | *oprN* | PJI74343·1 | 506 | 91% | 34% | NP251527 | 479 |
| **RND** | *oprM* | PJI74333·1 | 470 | 98% | 60% | NP249118 | 485 |
| **RND** | *mexB* | PJI74332·1 | 1049 | 99% | 64% | NP249117 | 1046 |
| **16S rRNA methyltransferase** | *rmtA* | PJI74285·1 | 332 | 99% | 71% | NP253317 | 332 |
| **RND** | *oprJ* | PJI74223·1 | 417 | 91% | 26% | NP251212 | 428 |
| **RND** | *mexY/H* | PJI74224·1 | 488 | 37% | 31% | NP253064 | 376 |
| **RND** | *mexW* | PJI74225·1 | 1052 | 98% | 36% | NP251210 | 1051 |
| **RND** | *mexW* | PJI74256·1 | 1053 | 99% | 78% | NP251210 | 1051 |
| **RND** | *mexV* | PJI74257·1 | 416 | 85% | 58% | NP251211 | 484 |
| **RND** | *opmH* | PJI74258·1 | 394 | 95% | 51% | NP251212 | 428 |
| **Porin** | *oprD* | PJI74259·1 | 447 | 99% | 58% | NP251195 | 448 |
| **16S rRNA methyltransferase** | *rsmG* | PJI74199·1 | 419 | 99% | 73% | NP255451 | 214 |
| **MFS** | *mdtN* | PJI74088·1 | 291 | 92% | 43% | NP252581 | 302 |
| **RND** | *oprM* | PJI74087·1 | 472 | 97% | 32% | NP251185 | 472 |
| **Porin** | *oprD* | PJI74021·1 | 444 | 99% | 48% | NP248982 | 460 |
| **Porin** | *oprD* | PJI73986·1 | 439 | 100% | 66% | NP251450 | 425 |
| **Porin** | *oprD* | PJI73790·1 | 446 | 99% | 43% | NP249649 | 443 |
| **RND** | *cusA* | PJI73799·1 | 417 | 96% | 49% | NP251212 | 428 |
| **RND** | *mexV* | PJI73798·1 | 399 | 85% | 68% | NP251211 | 484 |
| **RND** | *opmH* | PJI73797·1 | 1048 | 99% | 26% | NP251210 | 1051 |
| **Fluoroquinolone** | *gyrA* | PJI73676·1 | 921 | 99% | 86% | NP251858 | 923 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **ABC** | *macA* | PJI73394·1 | 961 | 98% | 98% | NP252924 | 945 |
| **23S rRNA methyltransferase** | *rlmN* | PJI73519·1 | 381 | 95% | 88% | NP252495 | 379 |
| **RND** | *mexN* | PJI73468·1 | 1030 | 98% | 72% | NP250127 | 1036 |
| **Porin** | *oprD* | PJI73464·1 | 444 | 100% | 60% | NP248852 | 444 |
| **RND** | *mexM* | PJI73602·1 | 387 | 99% | 48% | NP250126 | 385 |
| **Porin** | *oprD* | PJI73553·1 | 459 | 100% | 37% | NP249649 | 476 |
| **MFS** | *mdtG* | PJI73374·1 | 401 | 97% | 49% | NP250043 | 403 |
| **RND** | *mexY* | PJI73340·1 | 383 | 73% | 25% | NP253064 | 376 |
| **Porin** | *oprD* | PJI73206·1 | 422 | 100% | 52% | NP249649 | 443 |
| **Aminoglycoside** | *Aph* | PJI72935·1 | 339 | -- | -- | -- | -- |
| **RND** | *mexY/I/B* | PJI72905·1 | 791 | 100% | 84% | NP251769 | 793 |
| **Porin** | *ompA* | PJI72878·1 | 345 | 100% | 66% | NP250468 | 350 |
| **23S rRNA methyltransferase** | *rlmL* | PJI72886·1 | 730 | 100% | 81% | NP251738 | 725 |
| **RND** | *mexI* | PJI72862·1 | 1026 | 99% | 78% | NP252896 | 1029 |
| **RND** | *mexH* | PJI72861·1 | 365 | 98% | 54% | NP252895 | 370 |
| **Porin** | *oprD* | PJI72771·1 | 417 | 96% | 46% | NP249446 | 427 |
| **RND** | *muxC* | PJI72659·1 | 1035 | 100% | 59% | NP251216 | 1036 |
| **RND** | *muxB* | PJI72658·1 | 1032 | 98% | 72% | NP251217 | 1043 |
| **RND** | *muxA* | PJI72657·1 | 440 | 95% | 56% | NP251218 | 426 |
| **RND** | *opmB* | PJI72660·1 | 495 | 91% | 63% | NP251215 | 498 |
| **$\beta$-lactamase** | *bla*PDC-1 | PJI72364·1 | 380 | 93% | 52% | NP252799 | 397 |
| **Porin** | *oprD* | PJI72044·1 | 410 | 94% | 54% | NP252826 | 418 |
| **Porin** | *oprD* | PJI72100·1 | 416 | 99% | 48% | NP250903 | 416 |
| **23S rRNA methyltransferase** | *rlmL* | PJI71919·1 | 308 | 98% | 71% | NP250098 | 313 |
| **RND** | *opmH/opmB* | PJI71812·1 | 442 | 93% | 38% | NP252094 | 451 |
| **RND** | *oprM/oprJ/opmB* | PJI71694·1 | 423 | 98% | 92% | NP250566 | 425 |
| **RND** | *mexI* | PJI71718·1 | 821 | 96% | 49% | NP251769 | 793 |
| **ABC** | *macA* | PJI71650·1 | 390 | -- | -- | -- | -- |
| **ABC** | *macB* | PJI71651·1 | 654 | -- | -- | -- | -- |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **RND** | *oprM* | PJI71652·1 | 472 | 97% | 61% | NP251081 | 474 |
| **Sulfonamide resistance** | *sul1* | PJI71575·1 | 279 | -- | -- | -- | -- |
| **RND** | *mexE* | PJI71193·1 | 413 | 100% | 71% | NP251183 | 414 |
| **RND** | *mexF* | PJI71194·1 | 1059 | 100% | 92% | NP251184 | 1062 |
| **RND** | *oprN* | PJI71195·1 | 471 | 95% | 79% | NP251185 | 4772 |
| **Regulator** | *ompR* | PJI71217·1 | 233 | 99% | 51% | NP250490 | 235 |
| **RND** | *mexC* | PJI71218·1 | 391 | 91% | 51% | NP253289 | 387 |
| **RND** | *mexD* | PJI71219·1 | 1042 | 98% | 61% | NP253288 | 1043 |
| **MFS** | *emrA* | PJI71084·1 | 351 | 97% | 73% | NP251826 | 355 |
| **MFS** | *emrB* | PJI71085·1 | 516 | 95% | 85% | NP251827 | 499 |
| **PBP2** | *mrdA* | PJI70786·1 | 631 | 98% | 70% | NP252692 | 646 |
| **Aminoglycoside** | *aac(3)-iiia* | PJI70455·1 | 264 | -- | -- | -- | -- |
| **MFS** | *floR* | PJI70475·1 | 404 | 91% | 30% | NP252263 | 392 |
| **Transcriptional Regulator** | *tetR* | PJI70477·1 | 208 | -- | -- | -- | -- |
| **MFS** | *tetG* | PJI70478·1 | 391 | 35% | 48% | NP249822 | 422 |
| **Bleomycin binding protein** | *brp*MBL | PJI70471·1 | 121 | 98% | 36% | NP251817 | 267 |
| **β-Lactamase** | *bla*NDM-1 | PJI70472·1 | 270 | -- | -- | -- | -- |
| **Aminoglycoside** | *ant4-iib* | PJI70457·1 | 254 | -- | -- | -- | -- |
| **β-Lactamase** | *bla*PME-1 | PJI70444·1 | 309 | -- | -- | -- | -- |

These 98 antibiotics resistance genes are divided into two major groups: (i) the antibiotic efflux pumps (Table 4.5) and (ii) the enzymes that conferred antibiotic resistance (Table 4.6). Genes were also idetified in MR 02 that confer resistance to additional compounds (kanamycin, bleomycin). After annotations of these antibiotics resistance genes in different databases are nearly the same, we can suggest that the prediction results are more authentic.

Efflux pumps may be consist of single or multiple component, but in *Pseudomonas* many pumps have a three component systems and consist of inner membrane and outer membrane components as well as a membrane fusion protein (MFP) situated in the

periplasm. 64 genes are assigned to 21 efflux pump systems belonging to five different transporter families. 48 genes belong to resistance nodulation cell division (RND) family, 11 genes to major facilitator super family (MFS), single gene to multidrug and toxic compound extrusion (MATE) family, single gene to small multidrug resistance (SMR) family, and three genes to ATP-binding cassette (ABC) family (Table 4.5).

**Table 4.5: Predicted drug resistance-related porin channels and efflux pumps in *Pseudomonas* sp. MR 02**

| Porin channels and Multidrug transporter type | Gene locus |
|---|---|
| **Porin (OprD)** | PJI75663, PJI75717, PJI75483, PJI75048, PJI74259, PJI73986, PJI74021, PJI73790, PJI7334, PJI73553, PJI73206, PJI72771, PJI72100, PJI72044. |
| **RND efflux pumps** | PJI75714_75715_75716,PJI75773_75774,PJI75874,PJI75867_75868_75869,PJI75408_75409_75410,PJI74863,PJI74742_74743,PJI74580,PJI74343,PJI74333_PJI74332,PJI74223_74224_74225,PJI74256_74257_74258,PJI74087,PJI73797_73798_73799PJI73468,PJI73602,PJI73340,PJI72905,PJI72861_72862,PJI72657_72658_72659_72660,PJI71812,PJI71694,PJI71718,PJI71652,PJI71193_71194_71195,PJI71218_71219. |
| **MFS efflux pumps** | PJI75872, PJI75868_75869,PJI75712, PJI75788, PJI74088,PJI73374, PJI71084_71085, PJI70475, PJI70478. |
| **SMR efflux pumps** | PJI74577 |
| **MATE efflux pumps** | PJI75496 |
| **ABC efflux pump** | PJI71650_71651, PJI73394. |

14 homologues to the porin protein OprD and only one OmpA porin channel have been found Permeability barrier of outer membrane also play an important antibiotics resistance mechanism in *P. aeruginosa*. Low expression of *opr*D genes is responsible for making resistance against imipenem (carbapenem) antibiotics. Lack of OprD protein leads to reduction of active antibiotics molecules (Imipenem) capable of reaching the target penicillin-binding-protein (PBP) (Kadry, 2003). 14 *opr*D genes are identified in MR 02 (Table 4.5). All 14 *opr*D genes are mutated and unable to producing OprD protein.

Three different β-lactamase enzyme producing genes are present in MR 02. They belong to class A (PME-1 *P. aeruginosa* ESBL), class B (NDM-1), and class C (AMPC)

β-lactamase family (Table 4.6). First report of PME-1 came in 2011. It is grouped in Ambler class A extended-spectrum β-lactamase (ESBL), which is the most common cause of cephalosporin resistance in other Gram negative pathogens, such as *Escherichia coli* and *Klebsiella pneumonia*.

**Table 4.6: Predicted drug resistance-related enzymes in *Pseudomonas* sp. MR 02**

| Drug class | Putative product | Gene locus |
|---|---|---|
| **Beta-lactams** | Class A beta-lactamase | PJI70444 |
| | Class B beta-lactamase | PJI70472 |
| | Class C beta-lactamase | PJI72364 |
| **Aminoglycosides** | Aminoglycoside N-acetyltransferase | PJI70455 |
| | Aminoglycoside O-phosphotrnsferase | PJI72935 |
| | ANT(4) II family Aminoglycoside nucleotidyltransferase | PJI70457 |
| | 16S rRNA methyltransferase | PJI74285 |
| **Other** | Dihydrofolate reductase | PJI75361 |
| | 16S rRNA methyltransferase | PJI74199 |
| | 23S rRNA methyltransferase | PJI73519 |
| | 23S rRNA methyltransferase | PJI72886 |
| | 23S rRNA methyltransferase | PJI71919 |
| | DNA topoisomerase IV subunit A | PJI74590 |
| | Fluoroquinolone | PJI73676 |
| | Sulfonamied resistance | PJI71575 |
| | Fosfomycin thiol transferase | PJI72090 |

Eight genes responsible for enzymatic degradation of aminoglycosides antibiotics, three genes for aminoglycoside-modifying enzymes (AMEs) and five rRNA methylase genes (two 16S rRNA and three 23S rRNA) have been identified. AMEs attach a phosphate, adenyl or acetyl radical to the aminoglycosides antibiotic molecule, due to this attachments molecular structure of antibiotics are modified, and thus they decrease the

binding affinity of the antibiotics to the target in the bacterial cell (30S ribosomal subunit) (Llano-sotelo *et. al*., 2002). AMEs are divided into three classes: aminoglycoside phosphoryltransferases (APHs), aminoglycoside adenylyltransferases or nucleotidyltransferases (AADs or ANTs) and aminoglycoside acetyltransferases (AACs) (Vakulenko & Mobashery, 2003). On the basis of AMEs classification one AMEs from each group is presents in MR 02 (Table 4.6) and they make resistances against all types of aminoglycosides.

Microbes are resistance *Sul* 1 and *dfr* A genes responsible for mediating resistance against sulfamethoxazole and trimethoprim were also found in MR 02 genome. Dihydrofolate reductase resistance genes show 66% homology with *P. aeruginosa* PA01 whereas *sul* 1 gene is absent in PA01 genome.

Previous studies have shown the involvement of efflux channels and mutations of certain genes (*gyrA*, *gyrB*, *parC*, and *parE*) in the development of resistance to quinolone group of antibiotics in *Pseudomonas aeruginosa* (Nakajima *et. al*., 2002; Wang *et. al*., 2007). Mutated genes like *gyrA* and *parC are* also identified in MR 02 (Table 4.4 & 4.6). Presence of mutated genes like *gyrA* and *parC* along with the abovementioned efflux channels might have played a role in development of resistance to fluoroquinolone in MR 02.

We further analyzed the genomic islands by island viewer (Figure 4.1) and identified 14 antibiotic related genomic islands and 53 open reading frames. Three genes tetR PJI70477.1 (*tet*G regulatory protein) and two ABC efflux pump genes PJI71650_71651 precisely located in a putative genome island in the MR 02 strain, indicating that this gene was probably acquired through horizontal gene transfer; all the remnant genes were not present within any genomic island. One new RND efflux pumps PJI73799_73798_73797 are predicted, which is also located in putative genomic island. On the basis of these results suggest that the antibiotics resistance genes are conserved in *Pseudomonas* sp. MR 02 and are probably responsible for the intrinsic resistance in *Pseudomonas* sp. MR 02.

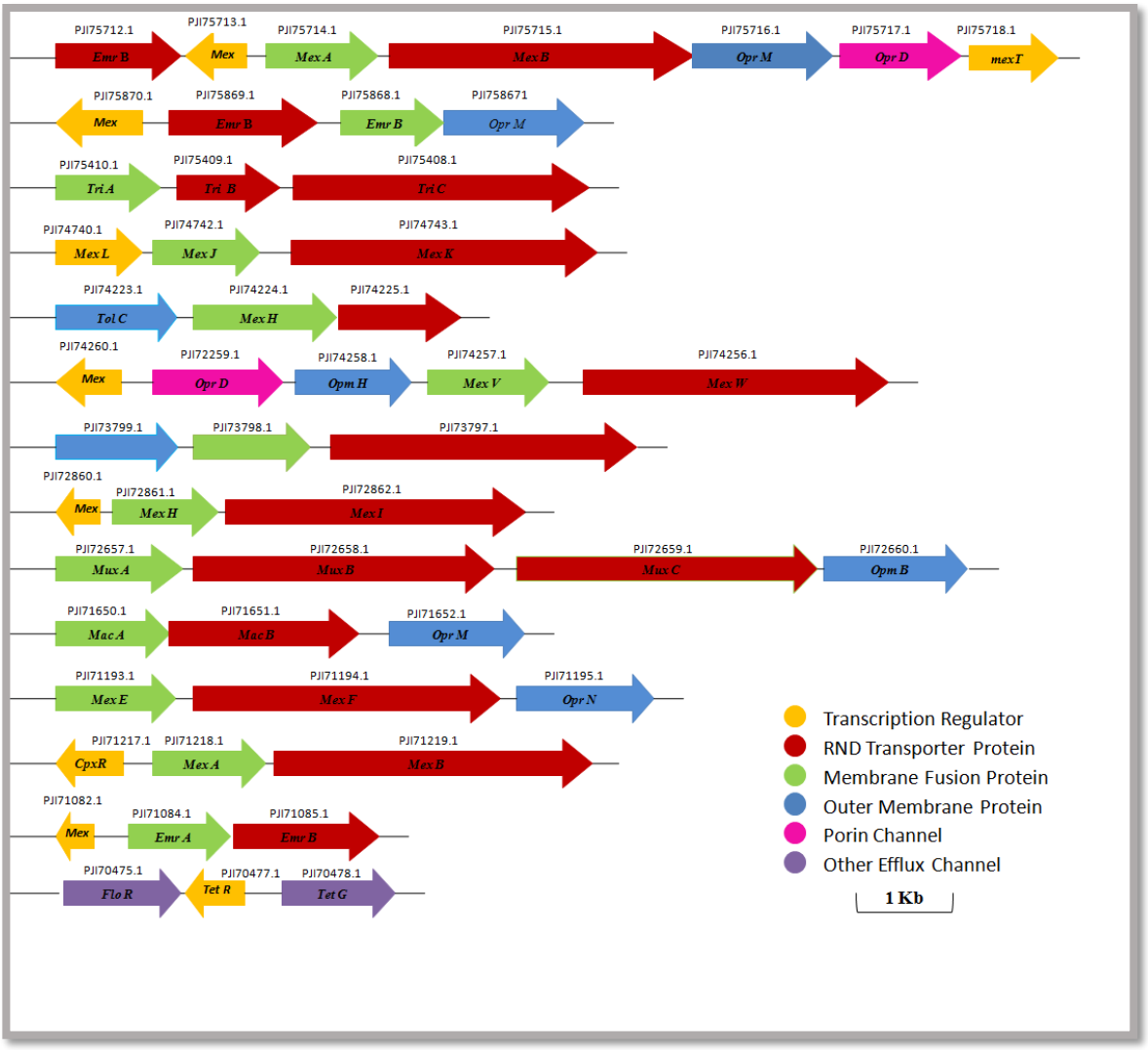


**Figure 4.3: A schematic representation of the cluster of antibiotic resistance gene in the draft genome of *Pseudomonas* sp. MR 02**

### 4.3.4 Taxonomic identification of *Pseudomonas* sp. MR 02

16S phylogeny revealed that strain MR 02 is a member of the genus *Pseudomonas*. Strain MR 02 revealed highest levels of sequence similarities with *Pseudomonas taiwanensis* DSM 21245 (99.7 %) followed by *Pseudomonas plecoglossicida* DSM 15088 (99.8%), *Pseudomonas monteilii* NBRC 103158 (99.7%) respectively. *Pseudomonas* sp. MR 02 was clustered in a separate branch with *Pseudomonas taiwanensis* DSM 21245 as its closest relative (Figure 2.6).

Though 16S rRNA is the unrivaled and universally applied marker of choice for most phylogenetic studies, but reliance on this single marker can result in poor resolution as there are several shortcomings in this approach. Genome based phylogeny plays a crucial role in phylogenetics of bacteria by replacing 16S phylogeny. Therefore the results of 16S phylogeny should be considered together with the results of whole genome based data analysis in a systematic process during species delineation. The bcgTree, (Figure 4.4), has revealed that the nearest phylogenomic neighbours of the strain MR 02 were *P. monteilii* CIP 104883, *Pseudomonas putida* NBRC 14164, *Pseudomonas hunanensis* P11.aa, *P. plecoglossicida* DSM 15088, *P. taiwanensis* DSM 21245, and *P. guariconensis* LMG 27394.

Two different *in silico* approaches were used in this study, *in silico* DDH and ANI, to determine the genomic similarities in order to confirm the species status. Both of these have been proposed as robust parameters (ANI value to define a new species is ≤ 96; and DDH value to define a new species is ≤ 70%) that can replace 16S rRNA gene sequence comparison for species delineation in the genomic era. The *i*DDH and ANI values obtained for MR 02 with *P. monteilii* CIP 104883 or *P. putida* NBRC 14164 or *P. hunanensis* P11.aa or *P. plecoglossicida* DSM 15088 or *P. taiwanensis* DSM 21245 or *P. guariconensis* LMG 27394 were 40.5 & 89.9% or 33.1 & 86.9% or 30.6 & 85.6% or 30.4 & 85.5% or 28.7 & 84.5% (Table A4.1 and A4.2). The analyses revealed that strain MR 02 showed maximum relatedness to the genome of the type strain of *P. monteilii*; values with other closely related type strains were even lower. These values qualify MR 02 strains as a distinct species of the genus *Pseudomonas* and different from any of their closest relatives.

The circular maps was used for gene characterization, functional assignment and function-based browsing of the MR 02 genome with 4 phylogenetically nearest species *P. plecoglossicida* DSM15088, *P. monteilii* NBRC103158, *P. putida* NBRC14164 and *P. aeruginosa* PA01. Non homologous regions present in the genome of four different nearest species of *Pseudomonas*, compared with MR 02 genome have been presented where a sub-set of it was related to antibiotic resistance genes. Total 8 genes were found unique to MR 02 (Figure 4.5).

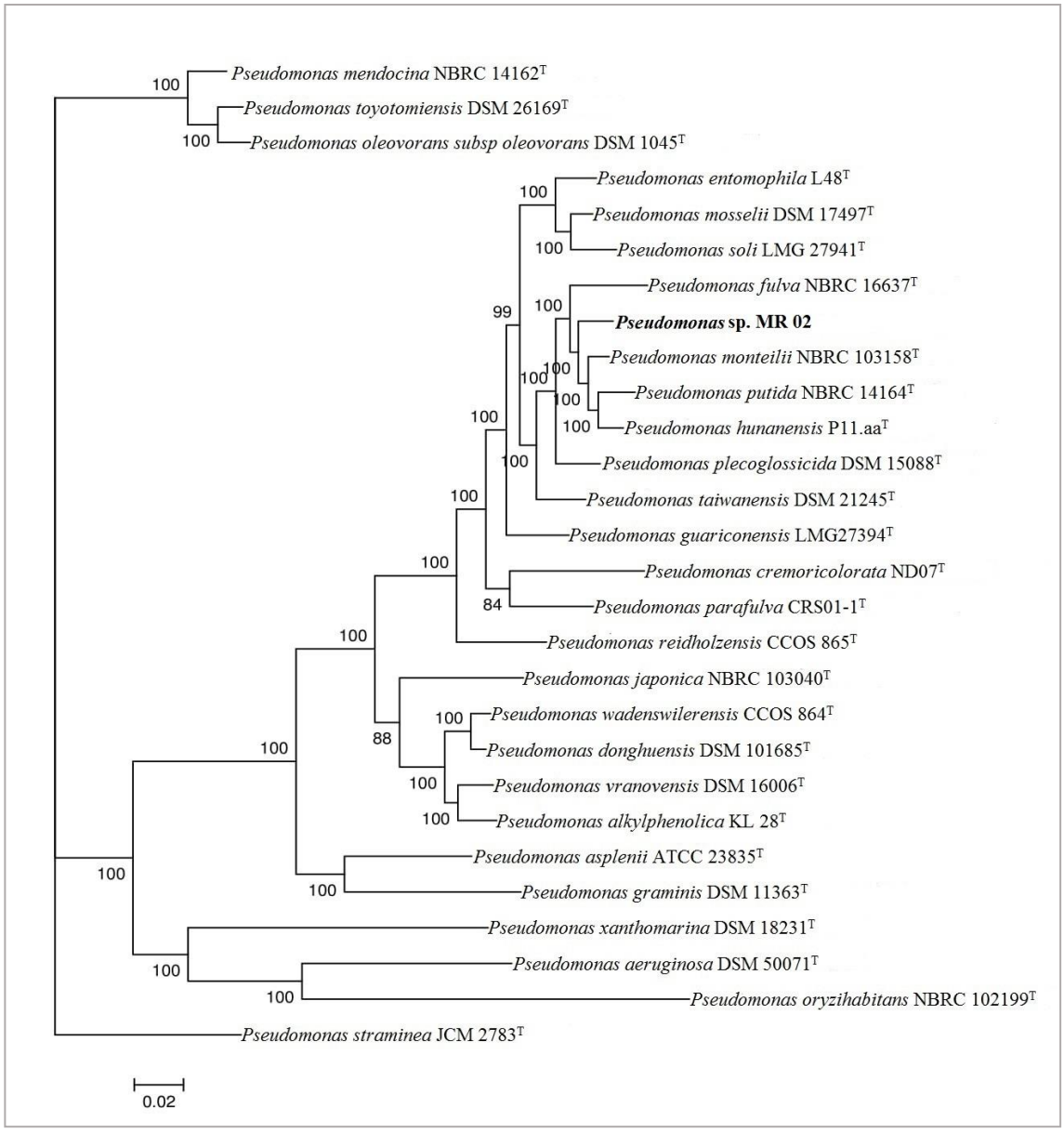


**Figure 4.4: A phylogenetic tree based on bacterial core genome**

The tree was built by the bcgTree pipeline for *Pseudomonas* sp. MR 02 along with 27 other *Pseudomonas* type strain genomes. Numbers at nodes designate bootstrap support values resulting from 100 bootstrap replicas.

## 4.3. Comparative genomic analysis of *Pseudomonas* sp. MR 02

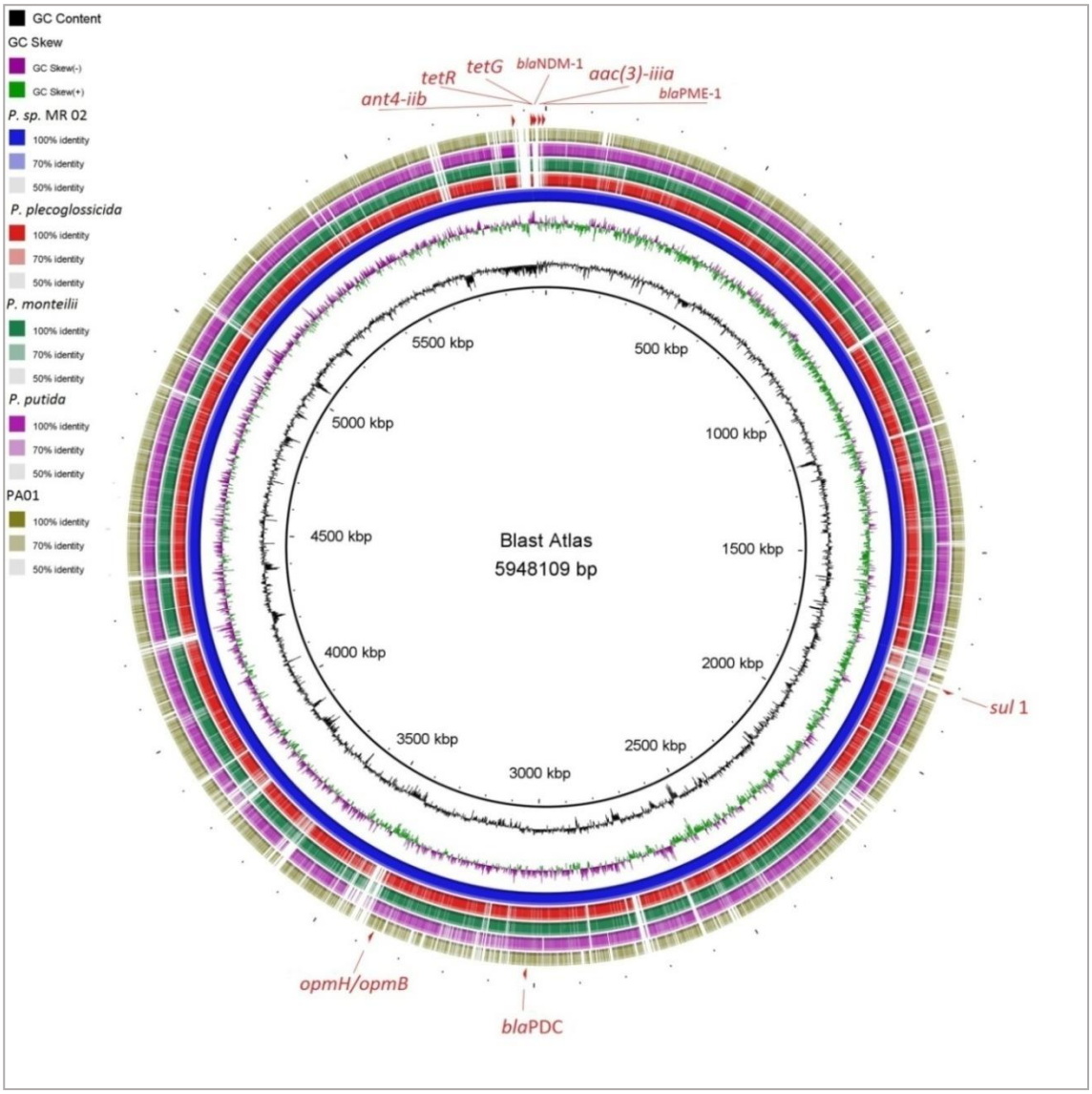


**Figure 4.5: Circular representation of the *Pseudomonas* sp. MR 02 draft genome in comparison with four reference genome of *Pseudomonas* genus**

Ring from inside to outside (1) GC contents (black) (2) GC skew (purple and green) (3) Coding sequences of *Pseudomonas* sp. MR 02 draft genome (dark blue) (4) BLAST comparison with *P. plecoglossicida* DSM 15088 (red) (5) BLAST comparison with *P. monteilii* NBRC 103158 (green) (6) BLAST comparison with *P. putida* NBRC14164 (violet) (7) BLAST comparison with *P. aeruginosa* PA01 (lime green) (8) Position of some important antibiotic resistance genes (red). Figure generated by BLAST Ring Image Generator v 0.95.

### 4.3.5 Correlation of phenotypic and genotypic characters of *Pseudomonas* sp. MR 02

Before the correlation of phenotypic characters of MR 02 with annotated 98 antibiotics resistance genes, we design two extra experiments: (i) determination of MIC value of MR 02 in presence or absence of PAβN (efflux pump activities determination), (ii) determination of MIC value of MR 02 in presence of EDTA (0.4 mM) (*bla*$_{\text{NDM-1}}$ activities determination) (Figure 1.10).

#### 4.3.5.1 Determination of efflux pump activity in contributing antibiotic-resistance in *Pseudomonas* sp. MR 02

In the presence of the efflux-inhibitor, PAβN, MICs of chloramphenicol, tetracycline, ciprofloxacin, and levofloxacin were reduced to 2.0, 2.0, 1.3, and 16.6 % respectively of the values shown in absence of PAβN (MIC values without PAβN was taken as 100%) , thus temporarily causing complete reversion of the resistance to susceptible phenotype. Similar reduction in MIC values, but of varying influence, has been observed for erythromycin (reduced to 20 %) and streptomycin (reduced to 33.3%) in presence of PAβN. The reductions in MICs, in presence of the efflux inhibitor, to lesser degrees were obtained with meropenem (reduced to 61.5%) imipenem (reduced to 75%), neomycin (reduced to 66.6%), gentamycin (reduced to 70%), cefepime (reduced to 75%) and ceftazidime (reduced to 75%). Least reduction in MIC in presence of PAβN was observed in case of trimethoprim or azithromycin (reduced to 83.3%) (Figure 4.6). Hence, on analyses, resistance shown by MR 02 against chloramphenicol, tetracycline, ciprofloxacin, and levofloxacin may be solely governed by the efflux pump(s) while resistance to other antibiotics except trimethoprim and azithromycin, were partially dependent on efflux system.

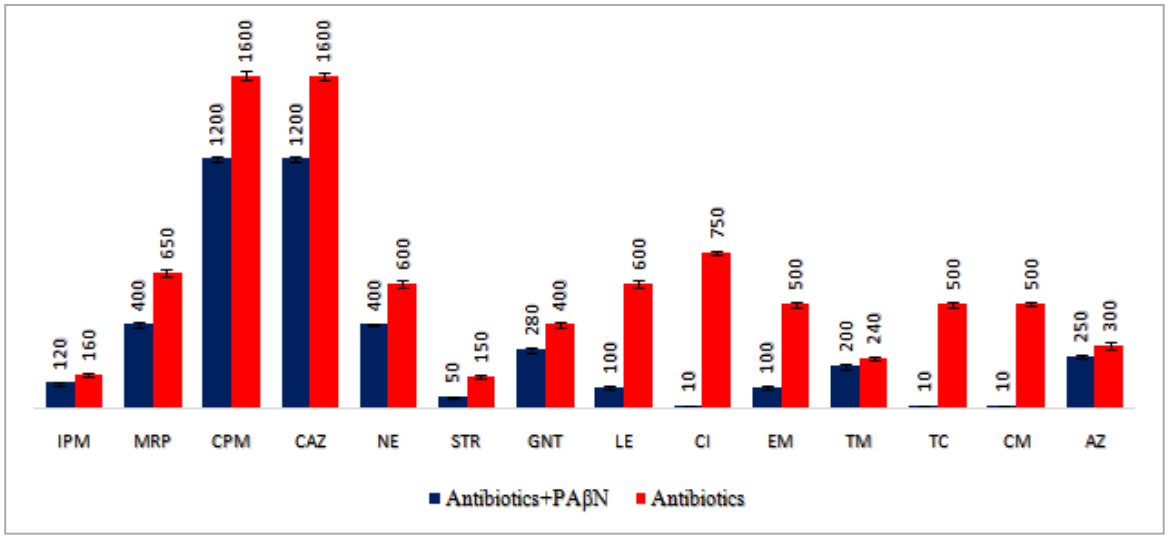


**Figure 4.6: Reduction in MIC values of *Pseudomonas* sp. MR 02 in presence and presence of PAβN with antibiotics**

MIC values of 14 antibioticsin the presence or absence of efflux pump inhibitor,PAβN (30µg/ml) ; MIC value expressed in µg/ml is shown at the tip of each bar graph. Neomycin(NE), Streptomycin (STR), Gentamycin(GNT), Trimethoprim(TM), Tetracycline(TC), Chloramphenicol(CM), Levofloxacin(LE), Ciprofloxacin(CI), Erythromycin(EM), Azithromycin (AZ).

Overall, phenotypic resistance pattern of MR 02 correlated highly with the predicted 98 resistance genes. Genotypic and phenotypic analysis showing only efflux pumps are responsible for resistance against chloramphenicol and tetracycline in MR 02 (Figure 4.6 and Figure 4.7). A comprehensive genomic distribution of antibiotics resistance genes in MR 02 annotated as per function to confer resistance to every single antibiotics of a total of 14 antibiotics (Figure 4.7). This resulted in an overall sensitivity of 100%. In this study, 0·4 mM EDTA was used to completely inhibit NDM-1 activity; which in turn reduced the MIC value of imipenem up to 95%. One metallo-β-lactamas genes encoding carbapenem resistance enzyme, and this results sensitivity was also 100%.

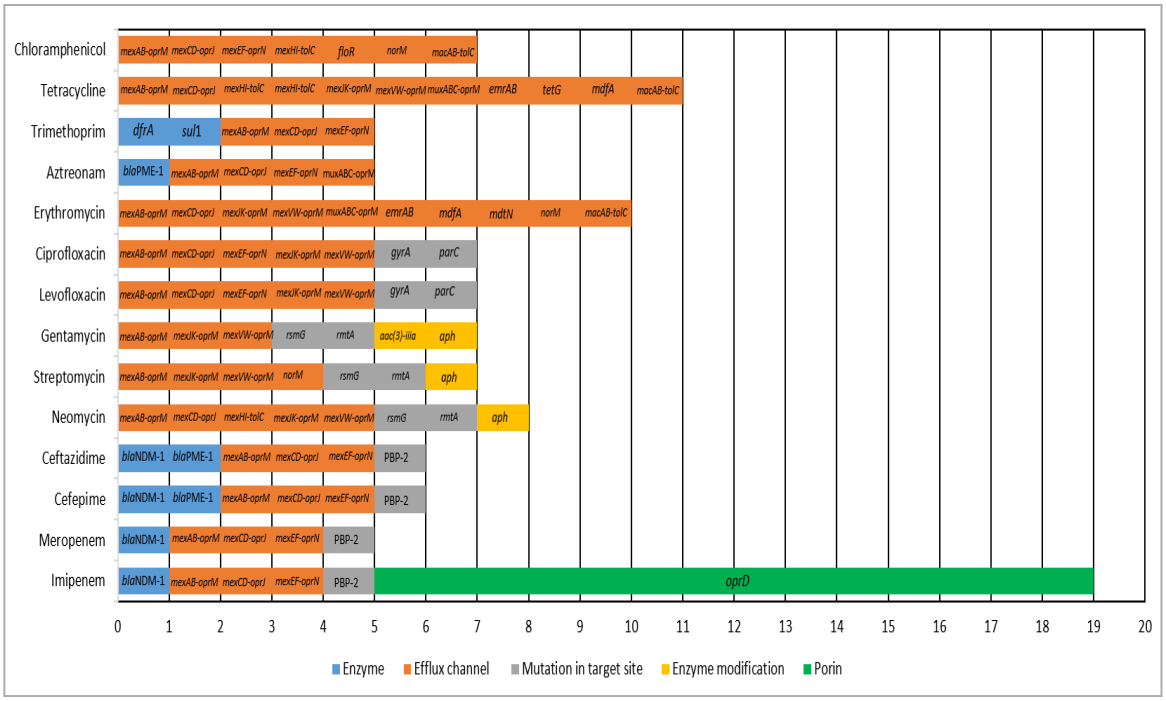


**Figure 4.7: A comprehensive genomic distribution of genes in MR 02 annotated as per function to confer resistance to every single antibiotic of a total of 14 antibiotics**

## 4.4 Conclusion

Whole genome sequencing in the current study was done to detect different types of resistance genes in *Pseudomonas* sp. MR 02 which were not detected in the previous chapters by phenotypic and PCR analysis. Whole genome sequencing helps in correlation between genotypic characters and phenotypic characters of *Pseudomonas* sp. MR 02. However, the antibiotic-resistance analysis tool (Resfinder, ARG-ANNOT, and CARD) was not efficient enough to detect the type of antibiotics resistance genes, while the BLAST tool (Smart BLAST) provided more detailed characterization of the ARGs. Although bacterial whole genomic studies are time consuming and expensive, they are more accurate and informative about the genetics of bacteria. Efforts are needed to make genomic study, and especially bioinformatics analysis, easier and more convenient for both laboratory and clinical and diagnostic work.

*Pseudomonas* sp. MR 02 strain carries chromosomally encoded complete package of resistance genes (efflux pumps, targets site modification, enzyme mortification, mutation in antibiotics binding sites, antibiotics degrading enzymes and non enzymic mechanism)

for aminoglycosides and β-lactams antibiotics. It has genotypically and phenotypically resistance to nearly all clinical trial antibiotics and they have lost susceptibility toward fluoroquinolone, aminoglycosides, monobactam, tetracycline, chloramphenicol, trimethoprim, colistin, cephalosporins, and carbapenem (including last resort antibiotic).

Detailed biochemical, physiological and phylogenetic analyses identified the strain MR 02 as a novel species of the genus *Pseudomonas* under *P. putida* group. In summary, MR 02 is a Pandrug-resistant bacteria and it has all possible arsenals arranged in its genome, ready for encountering representative molecules from any class of antibiotic.