

# Unique level of Multi-Drug Resistance (MDR) in *Pseudomonas aeruginosa* Strain DEB1

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**Abstract:** *Pseudomonas aeruginosa* is an opportunistic pathogen, a common cause of nosocomial infection having the ability to Multi-Drug Resistance (MDR). We have isolated the test bacterial strain from waste water. The isolated strain is identified by molecular marker method (16s rRNA sequencing) and *Pseudomonas aeruginosa* strain DEB1 (KF195926) has been deposited in Gene bank nucleotide sequence database. This article aims to summarize the antibiotic resistance profile (Disc diffusion method) of *P. aeruginosa* strain DEB1. It showed a unique level of natural MDR on twelve numbers of antibiotics. The study also focussed on the effect of temperature (4-60°C) and P<sup>H</sup> (5-9) on the growth pattern of this strain. *P. aeruginosa* is widely known for two distinctive properties: the production of the blue-green pigment, pyocyanin, and the ability to lyse or to inhibit the growth of other bacteria. This strain highly efficient to produce extracellular blue-green pigment within 24 hours in King'B medium. The study includes experiments to determine the effect of various media on pigment production.

**Keywords:** *P. aeruginosa*, MDR, Fluorescent, Pigment production.

## 1. Introduction

The opportunistic bacterial pathogen *P. aeruginosa* has received several names throughout its history based on the characteristic blue-green pigment production (Pitt, 1998). *P. aeruginosa* presents a serious therapeutic challenge for treatment of both community-acquired and nosocomial infections, and selection of the appropriate antibiotic to initiate therapy is essential to optimizing the clinical outcome (Bisbe et al., 1988; Micek et al., 2005). *P. aeruginosa* is a ubiquitous organism present in many diverse environmental settings, and it can be isolated from various living sources, including plants, animals, and humans (Bisbe et al., 1988). The ability of *P. aeruginosa* to survive on minimal nutritional requirements and to tolerate a variety of physical conditions has allowed this organism to persist in both community and hospital settings (Harris et al., 1984). In the hospital, *P. aeruginosa* can be isolated from a variety of sources, including respiratory therapy equipment, antiseptics, soap, sinks, mops, medicines, and physiotherapy and hydrotherapy pools (Pollack, 1995). Community reservoirs of this organism include swimming pools, whirlpools, hot tubs, contact lens solution, home humidifiers, soil and rhizosphere, and vegetables (Pitt, 1998; Pollack, 1995). The problem of antibiotic resistance in *P. aeruginosa* is on the increase (NNIS report, 2004). The heightened level of drug resistance is a result of the de novo emergence of resistance in a specific organism after exposure to antimicrobials (Carmeli et al., 1999) as well as of patient-to-patient spread of resistant organisms (Fridkin et al., 1999). Accumulation of resistance after exposure to various antibiotics and cross resistance between agents may result in multidrug-resistant (MDR) *P. aeruginosa* (D'Agata et al., 1997). Data from the National Nosocomial Infection Surveillance system from 1986-2003 reported *P. aeruginosa* as the second most common cause of pneumonia (18.1%), the third most common cause of urinary tract infection (16.3%) and the eighth most frequently isolated pathogen from the bloodstream (3.4%) (Gaynes et al., 2005). This condition was found primarily in patients with cystic fibrosis, where

persistent infection with *P. aeruginosa* leads to the sequential emergence of resistance to multiple antibiotic agents (Tenover et al., 1995). These MDR *P. aeruginosa* strains may be transmitted from patient to patient and sometimes lead to outbreaks among cystic fibrosis patients attending the same clinic (Saiman et al., 1996). This bacterium produces a pyocyanin (blue-green pigment). King, Ward, and Raney developed King A and King B agar media also referred to as *Pseudomonas* Agar P to increase pyocyanin production along with *Pseudomonas* Agar F to amplify fluorescein production (Abedon, 2009; Todar, 2008; Erica, 2010). In vitro, *P. aeruginosa* typically resembles a pearlescent color and is often foul-smelling (Qarah et al., 2010). To confirm diagnostically, *P. aeruginosa* DEB1 must possess either pyocyanin production or fluorescent production as well as the ability to grow at 37°C. This pathogen also is identified by its gram-negative rod-like appearance, a high positive oxidase reaction, and a lacking ability to ferment lactose.

## 2. Materials and Methods

### 2.1 Isolation of bacteria

The sample was collected from waste water (Sewage) of North Biswanathpur, East Medinipur, West Bengal-721439 India (Latitude-21.92475; Longitude-87.29335). The water sample was serially diluted (10-5) and 100µl (5plates) +120µl (5plates) of dilution samples was plated onto nutrient agar medium. After 48hours, we see that lightly greenish colony appeared. Pure culture was obtained by streaking 4-5 times in King's B medium. Then preservation for future we added 20% glycerol in pure culture and store at -200C Dubey (2007). For confirmation fresh samples were raised from glucerol stock and incubated for 48 h at 37°C with vigorous shaking at 200 rpm to provide aeration for the bacteria. A loopful of the resulting bacterial suspension was streaked onto asparagine plates containing 1.5% agar and incubated at 37°C until colonies developed. The bacterial isolate was then transferred to fresh asparagine plates according to the

morphological characteristics of colony: colour, shape and size (Al-Hinai et al., 2010). Standard staining procedures were undertaken.

## 2.2 Identification of bacteria

Comparison of rRNA gene sequences is currently considered to be the most powerful and accurate method for determining the degree to which microorganisms are phylogenetically related (Woese, 1987). The identity of bacterial isolate was confirmed using sequences of the 16S ribosomal RNA. DNA extraction, polymerase chain reaction (PCR) and sequencing were undertaken at Beckman Coulter (GenomeLab™ GeXP Genetic Analysis System), Applied Biostestem (VERITI) PCR AND MJ research PTC-200 PCR. The 16S ribosomal RNA region was sequenced using primers and compared using BLAST with sequences deposited at the National Center for Biotechnology Information (NCBI) (Larkin et al., 2007).

## 2.3 Outbreak of multi-drug resistance

Antibiotic resistance testing was determined by disc diffusion method (Himedia-IC002-IcosaG-1 plus) using Mueller-Hinton agar plates. The bacterial suspension were inoculated on the Mueller-Hinton agar plate by swabbing to give a smooth lawn, and antibiotic discs were placed on it, incubated at 37°C overnight.

## 2.4 Hydrogen sulfide production

Bacteria produced Hydrogen sulfide after degradation of cysteine or methionine which can be easily detected on Kligler's agar by formation a black precipitate at the stabbing side. It can be examined by using the P<sup>H</sup> indicator phenol red in the medium (Aneja, 2003).

## 2.5 Pigment and fluorescent production in various media

The first series of experiments was done to determine the effect of various media on pigment production. Nutrient extract broth was used as a base, and the medium was designated by whatever other ingredients were added: 1.0, 1.5, and 2.0 per cent glycerol (Young, 1947).

## 2.6 Effect on temperature

In order to find out the optimal conditions for growth of the bacterial isolate, the effect of different temperatures levels on the development and antagonism of antagonistic bacteria was examined. Three temperatures (28, 37, and 60°C) were mainly used (Al-Hinai et al., 2010). A bacterial suspension (100 µl) was placed in a tube containing 5 ml Kings's B broth. The tube was incubated at the predetermined temperatures for 48 h after which bacterial growth was determined by measuring the optical density (O.D.) at 660 nm (Ghosh et al., 1996).

## 2.6 Effect on p<sup>H</sup>

The effect of pH on bacterial growth was studied by using phosphate buffer with various pH values ranged from pH 5-9 (Gilbert et al., 1991).

## 2.8 Catalase activity

The purpose is to see if the microbe has catalase, a protective enzyme capable of destroying the dangerous chemical hydrogen peroxide. Growth from an overnight culture of the microbe is smeared on a microscope slide. A drop of 3% hydrogen peroxide is added (Rollins, 2009). If copious bubbles are observed, the microbe is positive for catalase.

## 2.9 Phenylalanine deaminase test

This test determines whether the microbe produces the enzyme phenylalanine deaminase, which is needed for it to use the amino acid phenylalanine as a carbon and energy source for growth. An inoculum from a pure culture is transferred aseptically to a sterile tube of phenylalanine agar to streak the slant. The butt of the tube need not be stabbed (Raducanescu et al. 1986). The inoculated tube is incubated at 37°C for 24 hours. After incubation, five drops of 10% ferric chloride and five drops of 0.1N HCl are added and the tube is gently shaken (Buiuc et al. 2009). A positive result is indicated if a green color develops within five minutes.

## 2.10 Indole test

Bacteria, which express the enzyme tryptophanase, can hydrolyze the amino acid tryptophan to indole, pyruvic acid and ammonia. Presence of indole can be shown by means of Kovác's reagent in the so-called indole test. Kovác's reagent contains p-dimethylaminobenzaldehyde, which forms a red complex with indole. Suspend one colony from a pure culture of the bacterium to be investigated, in a suitable medium. Incubate the medium at 37°C during 28 h. Add a few drops of Kovác's reagent (MacFaddin, 1980). Positive: The indole reagent change colour to cerise red. Negative: The indole reagent remains pale yellow.

## 2.11 Voges-Proskauer (VP) test

The VP test shows if the bacterium has butanediol fermentation and can split glucose to acetoin via pyruvate and further to 2, 3-butanediol (Dubey, 2007). If KOH (potassium hydroxide) is added, acetoin will be converted to diacetyl, which reacts with alpha-naphtol and forms a pink complex. Suspend one colony from the pure culture, which is to be investigated, in VP/MR medium (Mesaros et al. 2007). Incubate at 30-37°C for 24-48 h. Add 0.2 ml of 40% KOH and then 0.6 ml of alpha-naphtol solution. Positive: colour change to pink. Negative: no colour change.

## 3. Results and Discussion

### 3.1 Morphological, biochemical and molecular characterization of the isolated strain

The isolated was gram-negative, rod-shaped, Gammaproteo, acid-fast staining negative bacteria. Biochemical characterizations, the isolated strain was identified and remarked as belongs to the genus pseudomonas. By the molecular marker method (16s rRNA sequencing), confirmed the isolated as pseudomonas aeruginosa (using nBLAST-<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). In the phylogenetic tree, the strain was designated as P. aeruginosa

DEB1 and other closest *P. aeruginosa* strain were grouped together at a 99% similarity (fig1). The nucleotide sequence data of *P. aeruginosa* DEB1 has been deposited in the GeneBank nucleotide database under accession number KF195926.



**Figure: 1** Neighbors joining phylogenetic tree showing relationship between *P. aeruginosa* strain DEB1 (KF195926) with other isolates, based on their 16s ribosomal RNA sequence.

**Table: 1** Taxonomic characteristics of bacterial strain DEB1

Morphological characteristics	
Morphology : gram-negative, rod-shaped, acid-fast staining negative	
High growth medium : King's B & NAM	
pH : 5-9	Temperature : 28°C-60°C
Biochemical characteristics	
I. Indol test : Positive	VI. Peroxidase test : Positive
II. MR-VP test : Positive	VII. Phenylalanine deaminase: Positive
III. H <sub>2</sub> S Production : Positive	
IV. Catalase test : Positive	
V. Oxidase test : Positive	

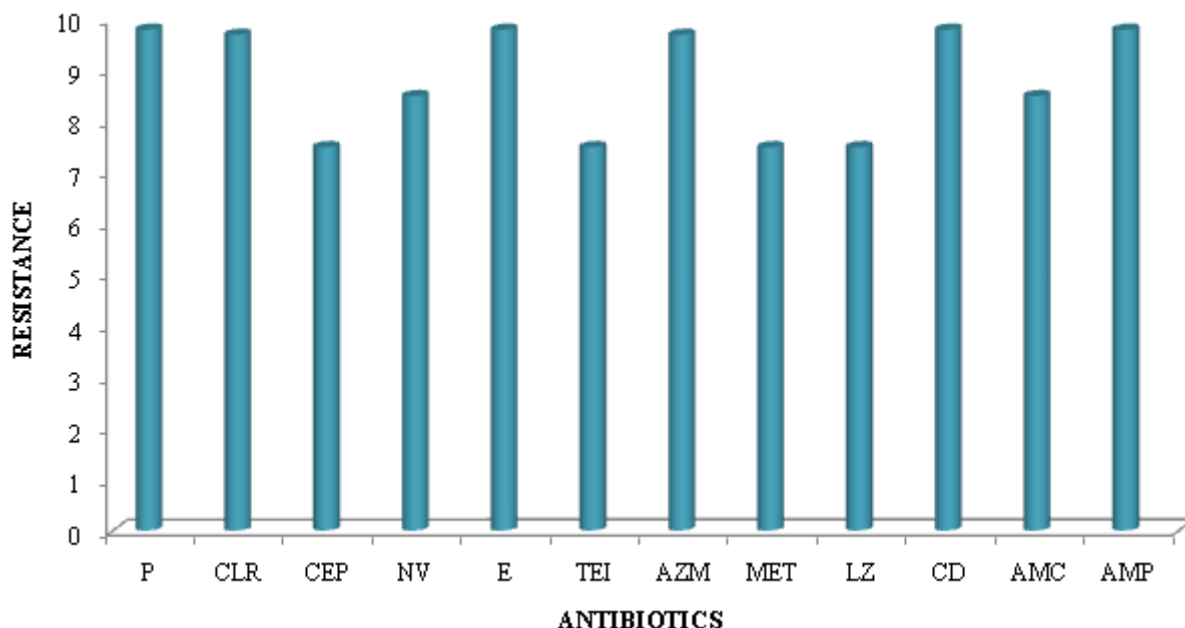
**3.2 Determination of Antibiotic Resistance Profile**

Isolated strain was resistant to twelve antibiotics (Tab-2 & fig-2). In that study isolated strain has a unique level of MDR on antibiotics.

**Table 2:** Antibiotic resistance activity of *P. aeruginosa* DEB1 after 24 hours

Sl. No.	Antibiotic Name	Used Antibiotics (µg)	Resistance Result
1.	Ampicillin	AMP10	R <sup>***</sup>
2.	Penicillin	P10	R <sup>***</sup>
3.	Clarithromycin	CLR15	R <sup>***</sup>
4.	Cephalothin	CEP30	R <sup>*</sup>
5.	Novobiocin	NV5	R <sup>**</sup>
6.	Erythromycin	E15	R <sup>***</sup>
7.	Teicoplanin	TEI10	R <sup>*</sup>
8.	Azithromycin	AZM15	R <sup>***</sup>
9.	Methicillin	MET5	R <sup>*</sup>
10.	Linezolid	LZ30	R <sup>*</sup>
11.	Clindamycin	CD2	R <sup>***</sup>
12.	Amoxycylav	AMC30	R <sup>**</sup>

**Legend:** R: Resistance; \*\*\*: Highlevel; \*\*: Mediumlevel; \*: Lowlevel



**Figure: 2** Antibiotic resistance activity of *P. aeruginosa* DEB1 (show in Clustered cylinder column)

**3.3 Blue-green pigments and fluorescence under Ultra Violet rays.**

Pyocyanin (from "pyocyaneus") refers to "blue pus", which is a characteristic of suppurative infections caused by *Pseudomonas aeruginosa* (King et al., 1954). *P. aeruginosa* DEB1 strain is highly producing pigment in various culture mediums (Tab-3).

**Table 3:** Pigment and Fluorescence production in various media by DEB1

Sl. No.	Used media	P <sup>H</sup>	Pigment	Fluorescent (UV light)
i.	King's B	7.2±0.2	+++	+++
ii.	NAM	6.8 ± 0.2	++	++
iii.	1.0%	7.5± 0.2	+	+
iv.	1.5%,	8.0± 0.2	++	++
v.	2.0%	8.2± 0.1	+	+
vi.	Medium A	7.0 ± 0.2	++	++
vii.	Blood Agar	7.3±0.2	++	++

**Legend:** +++: High; ++: Medium; +: Low

**3.4 Bacterial Growth Under Stress Condition**

The bacterial growth under stress condition was found to increase linearly until 2days at 4–60°C and pH 5–9. The growth was luxuriant (fig-4). Results revealed the potential of the strain to survive under stressful conditions (fig-3). The ability of the strain to adapt to temperature and pH stress may be important for the survival of the microorganisms during drought and different physiological conditions (Sasirekha et al. 2013).



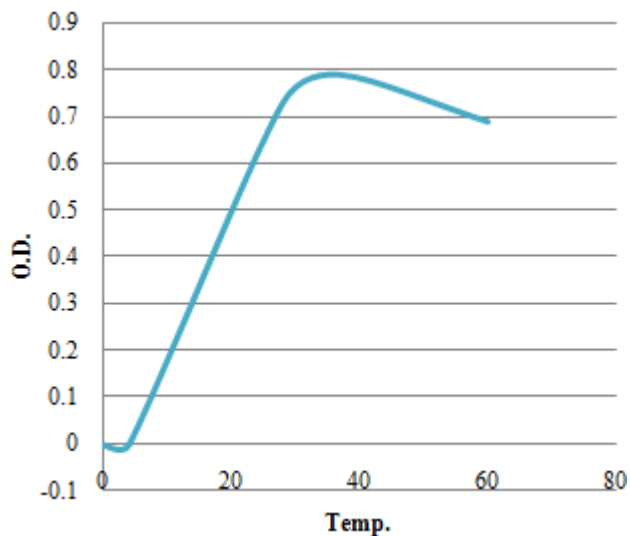


Figure: 3 DEB1 growths under different temperatures

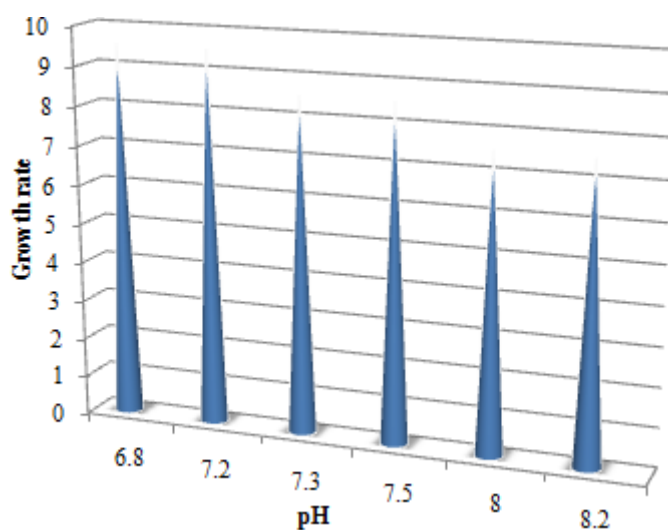


Figure: 4 DEB1 growths under pH

#### 4. Conclusion

The present study was an attempt to demonstrate the multifunctional property of *Pseudomonas aeruginosa* strain DEB1. *P. aeruginosa* strain DEB1 clearly represents one of the most challenging pathogenic bacteria. *P. aeruginosa* has been increasingly recognized for the ability to cause of infections. This report highlights the key role of sink design and patient room design in causing an pathogenic outbreak, and it outlines effective strategies to manage outbreak of this nature, though further investigations are needed.

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