

# Isolation of Multi-Drug Resistant *Paenibacillus* sp. from Fertile Soil: An Imminent Menace of Spreading Resistance

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**Abstract:** There are a good number of reports in the literature stating spread of resistance from normal soil flora to nosocomial microorganism through various ways. Similarly during the study of antimicrobial susceptibility pattern in the microflora, a multi-drug resistant bacterium has been isolated from soil collected Maharashtra state (India). The bacterium exhibited a resistance to various classes of antibiotics namely glycopeptide, beta-lactams, aminoglycosides, macrolides and lincosomides. The bacterial strain showed its resemblance to the genus *Paenibacillus*. This constitutes the first report of its kind as to the multi-drug resistance trait in the genus *Paenibacillus* phenotype, especially in close phylogenetic neighbour of *Paenibacillus daejeonensis*. The resistance pattern displayed by this strain particularly highlights the possible presence of multiple resistant determinants in microflora of the soil rhizosphere. In view of the recent reports about the *Paenibacillus* spp. in clinical derived strains, such multi-drug resistance factors in this genus adds to menace of transmission of resistance to common soil originating pathogen. The data also supports the fact that the resistances to certain antibiotics need not always be due to exposure to particular antibiotic or similar substance.

**Key words:** *Paenibacillus daejeonensis*, multi-drug resistant, *Paenibacillus*.

## 1. Introduction

The genus *Paenibacillus*, belonging to group III of the class Bacilli [1] has been reported from a variety of sources including soil, water, plants and insect larvae [2]. Majority of the bacilli of this group are all strict aerobes. Group III species are perhaps taxonomically the least satisfactory and are rather physiologically heterogeneous. The genus *Paenibacillus* does reflect physiological heterogeneity. *Paenibacillus* in its due course of time has been to have acquired resistance to the antibiotic Vancomycin. The presence of vancomycin resistance gene cluster in *Paenibacillus popilliae* homologous to *vanA* of Vancomycin

Resistant Enterococci (VRE) has been well reported [3]. *P. thiaminolyticus* and *P. apiarius* are also known to harbour putative D-Ala:D-Lac ligase genes flanked by *vanH* and *vanX*-like genes which are closely related to *van A* operon of Enterococci [4]. Recently widespread resistance to Oxytetracycline has been demonstrated in *P. larvae*, a primary pathogen of honey bees [5]. Different species of *Paenibacillus* seem to harbour resistance to different classes of antibiotics. *Paenibacillus sanguinis*, *Paenibacillus massiliensis* are already reported as atypical clinical isolates associated with Humans [6]. This probes as possible threat of spread of resistance into hospital derived pathogens through *Paenibacillus* spp.

In this investigation, the authors found a multi-drug resistant trait in *Paenibacillus* spp. strain MDR-1

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(Accession No. EU026352). The strain has been isolated from the fertile soil in the western region of the Maharashtra state (India). The strain is multi-resistant to existing principle antibiotics currently under use for the treatment of infections caused by both Gram positive and Gram-negative microbes.

## 2. Materials and Methods

### 2.1 Microbial Isolation

The soil samples were collected from agricultural field from Dahanu, a tiny village, 145 km north of Mumbai. Samples were collected from 0.6 - 1.0 feet depth of a quadrant at five different locations in the fertile fields and the samples were immediately transferred into sterile sampling containers. All the samples were transported to Piramal Life Sciences Limited (PLSL), Mumbai, India for bacterial isolation. The soil samples were processed within 24 hrs of collection. The samples were serially diluted using sterile saline and the appropriate dilutions were surface spreaded onto the isolation medium - Soybean Casein Digest Agar, SCDA (HiMedia, Mumbai) and incubated at 30 °C for 4 weeks. Amphotericin B (20 µg / ml) was incorporated into this medium to inhibit possible fungal growth that might occur during the prolonged incubation period. Macroscopic characteristics of the growing colonies were regularly monitored. The isolates were purified further and maintained on SCDA agar slants until used for further work. Colony characteristics were noted and cell morphology was examined by observing Gram stained specimens under oil immersion of light microscope. Motility was checked using hanging drop method [7]. Endospore staining was carried out according to Schaeffer-Fulton method [8].

### 2.2 Antibiotic Susceptibility Assay

Susceptibility to antimicrobial agents was determined by the standard Kirby-Bauer Disc Diffusion Method for all the isolates from that soil [9]. Twenty-five antibiotics viz. vancomycin, ampicillin,

carbenicillin, penicillin-G, cloxacillin, oxacillin, erythromycin, cephalothin, ceftriaxone, ceftazidime, cephalexin, gentamicin, netillin, amikacin, ciprofloxacin, nalidixic acid, ofloxacin, norfloxacin, lincomycin, clindamycin, co-trimazine, co-trimoxazole, nitrofurantoin, chloramphenicol, oxytetracycline, were chosen on the basis of their ability to provide diversity for representation of different antibiotic classes, which are currently under use. The soil isolates were grown in Soybean Casein Broth, SCB (HiMedia, Mumbai) for 18-20 hrs. The turbidity of the culture broth was adjusted to obtain absorbance of 0.3-0.45 at 560 nm. Mueller-Hinton medium agar was swabbed with the culture. The discs of mentioned 25 antibiotics (HiMedia, Mumbai) were placed in upright position on the surface of the test plates. After incubation of the plates at 37 °C for 18 - 24 hrs, the plates were scored for growth by measuring the diameter of the zone of inhibition with respect to each disc (6 mm diameter) by using a digital Vernier-Calliper. For the bacteria of the family *Bacillaceae* the zone of inhibition of  $\leq 12$  mm was considered resistant and  $\geq 35$  mm was considered sensitive [10]. As specifications for the zones between 12-35 mm were not available in The National Committee for Clinical Laboratory Standards (NCCLS) [10], antibiotics showing zones of inhibition in this range are recorded as not defined (N.D) [11]. *Enterococci faecium* ATCC19579, *Enterococci faecalis* ATCC 29212, *Enterococci faecalis* ATCC 47077 were used as quality control cultures [12]. All experiments were carried out in duplicate. The obtained multi-drug resistant isolate was further characterized by microscopic and macroscopic studies followed by phylogenetic analysis using sequencing of 16S rRNA gene by the method described elsewhere [13].

### 2.3 DNA Isolation

Cells were grown in 2-5 ml Soybean Casein Digest Broth till late log phase and pellet 4 ml cells in microfuge at 8,000 rpm for 10 min. The obtained pellet was resuspended in 200 µl Tris- EDTA buffer

containing 50 ng of RNase (Fermantas) and 400 µl of Sarkosyl solution (1% Sarkosyl, 0.5 M NaCl and 1% SDS) was incubated at 37 °C with intermittent shaking after every 5 min until the solution became clear. DNA was purified by using equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) and precipitated with 0.1 volume of sodium acetate (3 M) and 0.6 volume of isopropanol in microfuge at 8,000 rpm for 10 min. The obtained chromosomal DNA was washed with 500 µl of 70% ethanol for 10 min by spinning at 8,000 rpm and the sample was dried by placing open tube on bench top for 20 min and the resulting DNA was resuspended in 50 µl TE buffer. A Band for the corresponding DNA was observed on 1% Agarose gel prepared in 50 ml 1 X TAE (Tris-Acetate EDTA) buffer.

#### 2.4 PCR Conditions for 16S rRNA Gene Amplification

PCR amplification was performed for sample mixture of volume 50 µl containing the appropriate reaction buffer, reagents, the universal primer 27f (5'-GAGTTTGATCCTGGCTCA-3') and 1385r (5'-CGGTGTGTACAAGGCC-3'). The PCR conditions were as follows: initial denaturation (2 min at 95 °C), followed by 30 cycles of denaturation (1 min at 95 °C), primer annealing (1 min at 52 °C) and primer extension (1.5 min at 72 °C). Amplified gene products were visualized on 1% Agarose gel under UV at 254 nm.

#### 2.5 Blast and Phylogenetic Analysis

The PCR amplified product was purified using a Qiagen kit (No. 28104), and the product was used for sequencing on ABI 3700 Sequencer (MWG Bangalore, India). The gene sequences were analyzed against the NCBI database using BLAST N program packages and matched to known 16S rRNA gene sequences ([www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)).

### 3. Results

In this investigation, 33 bacterial strains were isolated

from the said soil samples. Among them, only one strain (Multi-Drug Resistant-1, MDR-1) showed resistance to 14 different antibiotics screened in cross-sectional *in-vitro* analysis (Table 1). Other 32 bacterial strains displayed sensitivity to most of the studied antibiotics (data not shown). For 10 antibiotics where zone sizes were in the range 12-35 mm there was no conclusive guidance available in the literature for interpretation.

Hence they were reported in the not defined (N.D) category. MDR-1 was sensitive to ciprofloxacin, a fluoroquinolone. The resistance pattern of the reference strains matched to their mentioned traits [12] (Table 2).

As revealed by the microscopic studies, the isolate MDR-1 was found to be gram-positive, rod-shaped and motile strain. The endospore staining experiment revealed the presence of centrally placed endospore in a swollen cell. The colony of strain MDR-1 was circular, off-white, effuse, opaque, and smooth on SCDA. The strain has been deposited at cell culture library at PLSL.

To further characterize the isolate, the 16S rRNA gene sequence analysis was performed. The 16S rRNA gene sequences obtained from the strain MDR-1 was then compared with those of previously studied *Paenibacillus* sp. The partial 16S rDNA sequence of the strain MDR-1 showed high similarity with *P. daejeonensis* (457 / 465, 98% similarity). The sequences have been submitted to Genebank (NCBI), and the information on which is available in an accession number "EU026352".

### 4. Discussions

*Paenibacillus daejeonensis* has been found to be Gram positive, rod shaped, centrally spored with swollen sporangium, motile bacilli. The microscopic characteristics of MDR-1 matched with *P. daejeonensis* producing circular, flat, smooth, opaque and white colonies on SCDA [14]. The strain exhibited high level of 16S rRNA gene sequence similarity to

**Table 1 Antibiotic resistance pattern of MDR-1 in antibiotics tested.**

Antibiotic class	Antibiotic tested	Disc conc. (mcg)	Zone Inh. Size (mm)	Interpretation
Glycopeptide	Vancomycin	30	0	R
	Ampicillin	10	0	R
	Carbenicillin	100	0	R
Beta Lactam	Penicillin-G	10U	0	R
	Cloxacillin	5	0	R
	Oxacillin	5	6.6	R
Macrolides	Erythromycin	15	0	R
	Cephalothin	30	0	R
Cephalosporins	Ceftriaxone	30	20.4	N.D
	Ceftazidime	30	16.8	N.D
	Cephodoxime	30	12.4	N.D
Aminoglycosides	Gentamicin	10	0	R
	Netillin	30	11.6	R
	Amikacin	30	0	R
Quinolones	Ciprofloxacin	30	40.6	S
	Nalidixic acid	30	16	N.D
	Ofloxacin	2	26.6	N.D
Lincosomides	Norfloxacin	10	28.8	N.D
	Lincomycin	2	0	R
	Clindamycin	2	0	R
Sulfonamides	Co-Trimazine	25	22	N.D
	Co-Trimoxazole	25	20.8	N.D
Nitrofurans	Nitrofurantoin	300	0	R
Chloramphenicol	Chloramphenicol	30	17	N.D
Tetracyclines	Oxytetracycline	30	16	N.D

R: resistant, S: sensitive, N.D: Not Defined, Inh: Inhibition, U : Units, Conc: Concentration.

**Table 2 Antibiotic resistance pattern of reference strains to Vancomycin.**

Reference strain	Antibiotic tested	Disc conc. (mcg)	Zone Inh. Size (mm)	Interpretation
E. faecium ATCC 19579	Vancomycin	10	8	R
E. faecalis ATCC 29212	Vancomycin	30	22	S
E. faecalis ATCC 47077	Vancomycin	30	28	S

*Paenibacillus daejeonensis* and other close phylogenetic neighbors were *P. agaridevorans* and *P. illinoisensis*.

In the past, vancomycin and oxytetracycline resistance has been demonstrated by many species of *Paenibacillus* [3, 5] but no antibiotic resistance has been reported in *Paenibacillus daejeonensis*. However, in the present investigation, *P. daejeonensis* demonstrated resistance to vancomycin, ampicillin,

carbenicillin, penicillin-G, cloxacillin, oxacillin, erythromycin, cephalothin, gentamicin, netillin, amikacin, lincomycin, clindamycin, and nitrofurantoin. This work constitutes the first report of its kind as to the robust multi drug resistance trait reported ever in a *Paenibacillus* spp. The *van* operon responsible to vancomycin resistance in this genus was found particularly similar to that in enterococci, suggesting that the dissemination of glycopeptides resistance may

have occurred via mobile elements present in soil resistome [15]. The present *P. daejeonensis* strain has shown resistance to the large classes of antibiotics viz Beta lactams, aminoglycosides, macrolides, cephalosporines and lincosomides. The resistance pattern displayed by this strain highlights the possible presence of multiple resistant determinants in microflora of the soil rhizosphere in particular. And this data specifically points out the extent of overuse of potential life saving antibiotics, which have resulted in development, and conservation of resistance in non-pathogenic microorganisms, such as *P. daejeonensis*. A report published in the year [16] 2003 highlights points on resistance in bacteria and discuss in depth the plethora of mechanisms involved, including several that have only recently been discovered [16]. The report also describes the existence of number of elements in the epidemiology of bacterial resistance that have not been fully understood yet. However the report failed to explain the reason why certain multidrug-resistant bacteria spread widely and others not [16] Moreover it should be noted that *Paenibacillus*, being an atypical pathogen of human and animals, should never have been exposed to an antibiotic as such except those produced in soil micro-flora. Chances of exposure of this genus to multiple antibiotics are also remote. In this regards such multi-drug resistance factors in *Paenibacillus* add intimidation of transmission of resistance to common soil originating pathogens.

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