

# Arsenic-induced antibiotic response in bacteria isolated from an arsenic resistance estuary

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## ABSTRACT

The objective of the study is to determine the antibiotic resistance (AR) of bacteria in the presence of arsenic (As). AR profile was estimated by growth curve analysis and its morphological alterations were recorded using a field emission scanning electron microscope (FESEM). Preliminary screening of the isolates revealed that PS-6 showed the highest As resistances at 1,000 mg l<sup>-1</sup> concentration which was identified as *Bacillus licheniformis* using 16S RNA analysis. In the bacterium–As–antibiotic interaction, the culture showed sensitivity toward antibiotics in the initial phase but after treatment with inorganic As (V) at 50 mg/l, the bacterium developed resistance toward antibiotics like ampicillin, erythromycin, and methicillin with 38%, 30%, and 91%, respectively. In case of Chloramphenicol (C) and Kanamycin (K), a non-significant difference in the zone of clearance was observed indicating a reduction in the bacterial growth in the presence of erythromycin, whereas in the presence of As it increased. FESEM analysis showed clumping and aggregation in As-treated and As–erythromycin-treated cells denoting the AR. These results eventually state that the bacterium exhibit an adaptive mechanism to overcome antibiotic stress influenced by As, which opens a new window in understanding the role of metalloids in AR and its adaptive pathway.

## 1. INTRODUCTION

Arsenic (As) is one of the well-known toxic chemicals that was listed in the US Comprehensive Environment Response, Compensation, and Liability Act of hazardous substances [1]. The level of toxicity and the extensive contamination of the As are alarming issues as the metalloid does not degrade, nor can it be destroyed in the environment. As in the atmosphere enters into the human system through ingestion and/or inhalation. Ingestion of As causes symptoms of acute gastrointestinal irritation, whereas inhalation of As leads to major respiratory illness. The half-life time of ingested As is shorter than inhaled As due to more rapid biotransformation in the liver [2]. However, absorption of As leads to a wide range of probable symptoms that in turn reflects on organ damages. As the mobilization and contamination of As in the environment and human system is becoming a serious global

issue, methods to remediate As toxicity have received increasing international attention.

Antibiotic resistance (AR) is an adaptive mechanism preferred by bacterium to conquer the antibiotic stress in order to retain its survival. Initial adaptation of the bacterial AR starts with formation of the biofilm that restricts the interaction of antibiotics with the bacterial system [3]. The most common reason for the development of AR in bacteria was over usage of antibiotics, heavy metal cocontamination, disposal of expired antibiotics, and delay in development of new antibiotics [4,5]. Hence, increasing AR among the normal flora and opportunistic pathogens is considered a major threat to the human population [6]. The development of resistance genes among the natural ecosystem keeps changing between the population and within species [7]. Bacteria belonging to the same family habituated in a different environment like the deep sea, terrestrial, and ice surface possess varying levels of AR genes in their genome [8,9]. The excess usage of pesticides containing heavy metal induces heavy metal resistance, subsequently leading to the resistance of antibiotics like ampicillin and tetracycline in bacteria [10]. The evidence for

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the contribution of AR from the natural ecosystem to the clinically relevant microorganism is not clearly stated [11]. Accepting and acquiring the AR gene from the environment through plasmids, cosmids, transduction, transposons, and mutation are the mere possible ways of developing AR in bacteria [12]. Antibiotic gene transportation from vertebrates and abiotic contacts like groundwater, surface flow, and aerial dispersal are also possible [13]. Cadmium and nickel-resistant bacteria are more resistant to ampicillin and chloramphenicol [14]. A diversified mode of action by antibiotic and common efflux mechanism by bacteria could be the possible reason for the coselection and tolerance development in bacteria [13]. The contribution level of heavy metals and biocides in the development of AR genes is not clearly understood due to the lack of experimental evidence [15]. Lack of basic knowledge in understanding the biochemical and physiological mechanism of resistance is the primary reason for the failure of controlling AR in the environment [10]. Among the commensals, *Escherichia coli*, *Klebsiella*, and *Pseudomonas* spp. are considered as indicator organisms for assessment and monitoring of AR in the environment and clinical samples [16-18]. The gap in understating the role of heavy metals in AR always coexists with the mechanism of AR acquisition in the bacterial systems. The present hypothesis is to predict the correlation of a heavy metal (As) and its influence in the development of AR in the isolated culture of estuary sediments from Pichavaram mangrove.

## 2. MATERIALS AND METHODS

### 2.1. Sample Collection

Sediments were collected from the Pichavaram mangrove estuary, Tamil Nadu, India. The surface soil with 0–5 cm depth was collected in polythene bags, sealed and stored at 4°C until further study. The serially diluted sediment was plated on nutrient agar medium and incubated at 37°C for 72 hours for isolation of bacterial culture [19].

### 2.2. Identification of the Study Organism

The isolated bacterium was determined for its As tolerance potential by varying the concentrations (100–1,000 mg l<sup>-1</sup>) of sodium arsenite [As (III)] or sodium arsenate [As (V)]. Based on the initial screening, seven bacteria showed tolerance to inorganic As (III) and As (V) (Table 1). Among which bacterium PS06 exhibited highest As tolerance potential up to 1,000 mg l<sup>-1</sup> for inorganic As (III) and As (V) and it was selected for further identification. Based on the phenotypic and genotypic identification by 16S rRNA sequencing [20], the bacterium PS06 was identified as *Bacillus licheniformis* and a gene sequence was deposited in NCBI (Accession No: KJ933861)

### 2.3. Antibiotic Profile of *B. licheniformis*

Antibiotic response of *B. licheniformis* was studied based on the methods described earlier [21]. In brief, a uniform lawn of log phase culture equivalent to a population of 10<sup>8</sup> cells was prepared in the pre-molten Mueller–Hinton plates (Himedia, India). Pre-coated commercial antibiotic disks (Himedia, India) six disks

**Table 1:** Arsenic tolerance potential of Pichavaram isolates.

S. No	Strain ID	Arsenic tolerance concentration (mg l <sup>-1</sup> )	
		As (III)	As (V)
1.	PS-1	200	600
2.	PS-2	400	700
3.	PS-3	–	100
4.	PS-4	–	200
5.	PS-5	–	100
6.	PS-6	1,000	1,000
7.	PS-8	300	700

“–” indicates no growth was observed after incubation.

**Table 2:** Pattern of arsenic-induced AR in *B. licheniformis*.

S. No	Antibiotic used	Reference <sup>a</sup>	Zone of Inhibition in mm		
			Control	As (III)-treated 50 mg l <sup>-1</sup>	As (V)-treated 50 mg l <sup>-1</sup>
1.	AMP <sup>10</sup>	13	18 ± 0.5	12.3 ± 0.57 <sup>b</sup>	11.0 ± 0.1 <sup>b</sup>
2.	C <sup>30</sup>	12	18 ± 0.5	15.6 ± 0.57	22.0 ± 0.33 <sup>c</sup>
3.	CF <sup>5</sup>	15	27 ± 0.5	42.0 ± 1 <sup>c</sup>	41.0 ± 0.2 <sup>c</sup>
4.	G <sup>10</sup>	12	19 ± 0.1	26.6 ± 1.15 <sup>c</sup>	26.0 ± 0.4 <sup>c</sup>
5.	K <sup>30</sup>	13	20 ± 0.2	24.6 ± 0.5	21.0 ± 0.01
6.	Met <sup>5</sup>	9	23 ± 0.6	0 <sup>b</sup>	0 <sup>b</sup>
7.	Ery <sup>15</sup>	14	17 ± 0.3	11.0 ± 1 <sup>b</sup>	10.0 ± 0.1 <sup>b</sup>

Values in superscript denote the concentration of antibiotics (mcg/disk).

AMP = Ampicillin; C = Chloramphenicol; CF = Ciprofloxacin; G = Gentamicin; K = Kanamycin; Met = Methicillin; Ery = Erythromycin.

<sup>a</sup>Represents size mentioned for sensitivity in zone size interpretative chart Himedia.

<sup>b</sup>Resistance toward As treatment.

<sup>c</sup>Sensitive toward As treatment.

were placed at regular intervals and incubated inverted at 37°C for 24 hours. Bacterial cells grown in the medium containing 50 mg l<sup>-1</sup> of As (III) and As (V) were used to study the alteration in antibiotic pattern between untreated cells. Antibiotics used in the study are described in Table 2.

### 2.4. As-Induced Antibiotic Profile in *B. licheniformis*

Based on the disk diffusion assay, erythromycin was chosen to study growth response and structural modification upon As and erythromycin treatment. *Bacillus licheniformis* was grown in nutrient broth containing 50 mg l<sup>-1</sup> of As (V) and erythromycin. A flask containing 50 and 100 mg l<sup>-1</sup> of As (V) and 10 and 50 mg l<sup>-1</sup> of erythromycin was used as reference. The growth rate of the bacterium was monitored by measuring its turbidity. Samples were withdrawn aseptically at 10-hour intervals and analyzed using a spectrophotometer at OD<sub>600</sub> nm.

### 2.5. Field Emission Scanning Electron Microscopy

The bacterial cells were collected by low-speed centrifugation without disruption and were fixed with 1% glutaraldehyde. The cells were washed thoroughly using sterile PBS thrice to remove the unbound glutaraldehyde before mounting on to the aluminum stub and sputtered with gold particles. Samples treated with As (V), erythromycin, and both were analyzed separately. Untreated bacterium was used as control to understand the

morphological variation that aroused after treatment with As and erythromycin

### 3. RESULTS AND DISCUSSION

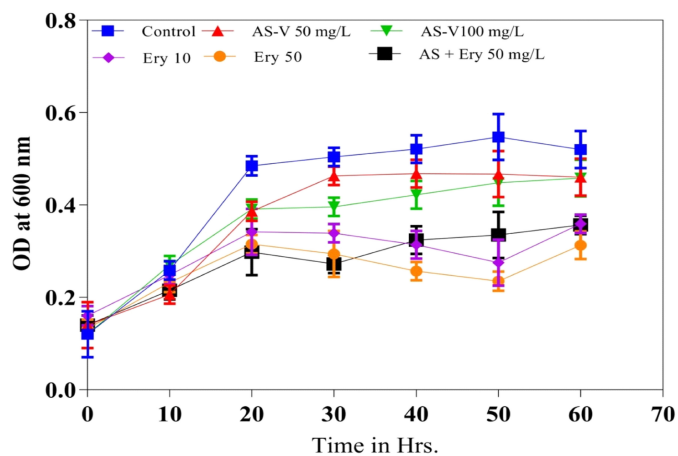
Studies relating to the nature of As and its tolerance are needed to address environmental issues. Table S1 demonstrates the properties of the sediments using inductively coupled plasma-atomic emission spectroscopy (ICP-OES) where the sample appeared to be fine and silky, with a powdery appearance with silicon, calcium, iron, and aluminum as main constituents. The fine powdery appearance of the sediment is due to the higher concentration of silica and the neutral pH of the sediment soil favors the growth of neutrophilic bacteria [22].

Microbiological screening of sediments revealed that very few bacterial isolates were able to grow in the plate containing As. Seven bacterial species were isolated from the sediments of Pichavaram estuarine (Table 1) which showed growth potential for various concentrations of As (III) or As (V). From this, PS-6 was chosen for the present study based on its higher tolerance up to 1,000 mg l<sup>-1</sup> for the inorganic As (III) and As (V) (Table 1). Other isolates, such as PS-3, PS-4, and PS-5, were identified as As resistance bacteria of As (III) since no growth was observed in the plates. The ability of the bacteria to tolerate a higher concentration of As from the As source is well reported [23]. The bacterium PS-6 showed potential As tolerance, irrespective of its origin from As-free environment, which was confirmed using ICP-OES (Table S1). Partial 16S rRNA sequencing revealed that As-tolerant isolate PS-6 was identified to be *B. licheniformis*.

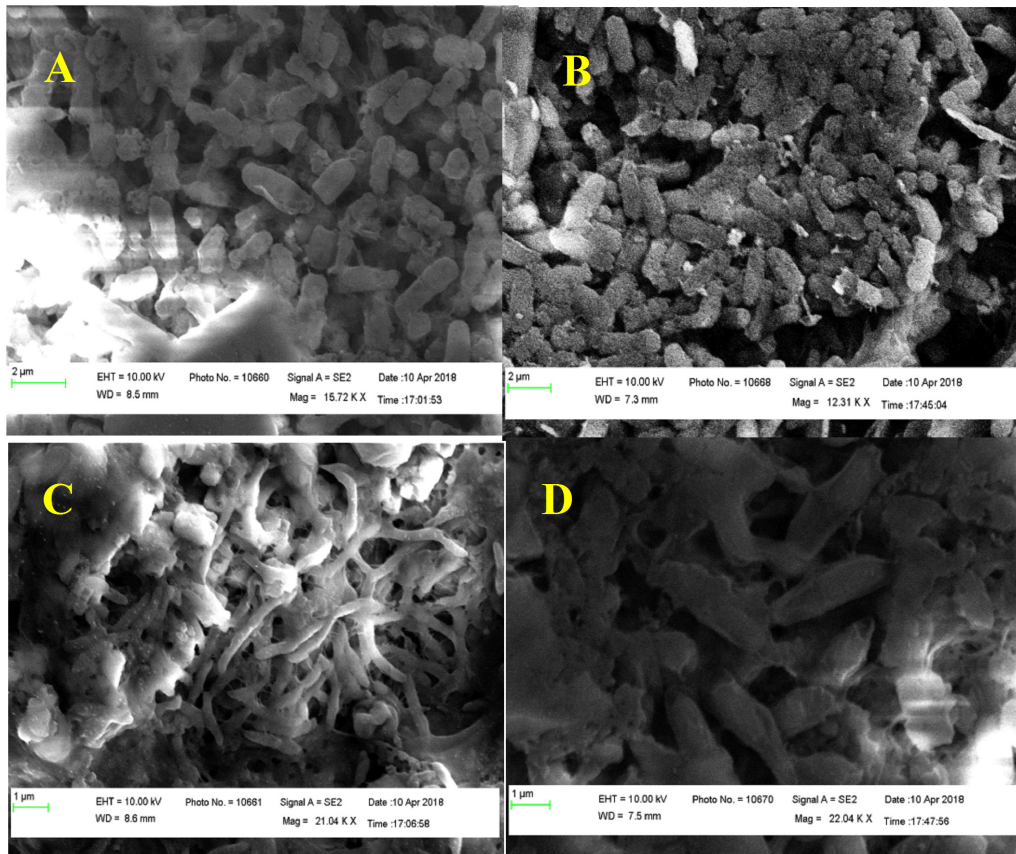
Development of heavy metal-driven AR with reference to heavy metal source and toxicity was discussed earlier [24]. The results showed that in the absence of As, *B. licheniformis* showed sensitivity to the entire antibiotic group used in the study. However, in the presence of As (V) treatment, *B. licheniformis* showed development in AR toward ampicillin (35%), erythromycin (24%), and methicillin (91%). A similar resistance pattern was observed with As (III)-treated *B. licheniformis* exhibiting ampicillin, erythromycin, and methicillin at 32%, 39%, and 91%, respectively. In addition, As-treated *B. licheniformis* was susceptible to ciprofloxacin up to 34% for As (V) and 36% for As (III) treatment. In gentamicin, it was 27% for As (V) and 27% for As (III); in chloramphenicol, it was 22% for As (V) and 11% for As (III), whereas in Kanamycin, it was 4.7% for As (V) and 17% for As (III) (Table 2). This evidence supports the AR/sensitivity development of heavy metals among the bacteria from the As-free environment. Chen et al. [25] reported the AR toward tetracycline by the As-tolerant bacterium LSJC7. The above results were found to be in accordance with previous studies [14], wherein minimum concentration of heavy metal was sufficient for induction of AR in soil bacterium [10,24]. In preliminary screening, both As (V) and As (III) were checked for AR. Since As (V) was found to be involved more in human interactions [26], further studies on growth kinetics and field emission scanning electron microscope (FESEM) will be determined using As (V) in *B. licheniformis*.

The result of the growth curve indicates that As at 50–100 mg/l concentration, the growth of the organism was not restricted, which was similar to the control. In treatment with erythromycin, the growth of the organism decreased after 30 hours. In case of As (V) (50 mg/l) with erythromycin, the growth pattern of *B. licheniformis* was significantly improved after 30 hours in comparison with the organism treated with erythromycin alone (Fig. 1). These results indicate the acceptance of erythromycin resistance by As. Liu et al. [27] reported the ability of antibiotics in physiological adaptation, wherein macrolides antibiotics like erythromycin was shown to have better inductive ability than other aminoglycoside antibiotics. Previous study on the development of AR in ureolytic bacteria showed relevant correlation with influence of heavy metal [28]. Resistance toward antibiotics in the As-treated culture may be due to the induction and/or coexpression of biocidal and metal resistance genes [14,29]. A similar study on the prevalence of tetracycline resistance among *E. coil* in Tagus estuary was also reported [29] which is in accordance with our results.

Based on the growth pattern (Fig. 1), an attempt was made to check any morphological alteration in *B. licheniformis* caused by As and erythromycin. FESEM analysis showed significant variation, which confirms the influence of As and antibiotics on the cell surface. Protrusion of a bacterial cell wall and pleomorphic variation was recorded in As-treated and As +erythromycin-treated *B. licheniformis* (Fig. 2). Protrusion was the mechanism of bacteria used during transfection and interaction of cationic ions like silica [30]. Mohanty and Mishra [31] reported that the influence of silica on *B. licheniformis* causes pleomorphic modification. These types of pleomorphic forms are not an artifact but they represent various levels of a life cycle in stressed bacteria [32]. Therefore, these results state that change in extracellular modification in turns alters the intracellular metabolism of the cell. Hence, modification in kinetics of growth and pleomorphic variation of *B. licheniformis* strongly indicate the influence of As in induction of resistance to erythromycin and other antibiotics.



**Figure 1:** Growth curve of *B. licheniformis* with arsenic As (V) 50, and 100 mg/l, erythromycin 10 and, 50 mg/l, and arsenic As (V) and erythromycin 50 mg/l.



**Figure 2:** Field emission scanning electron micrograph of *B. licheniformis*. (A) Control, (B) erythromycin 50 mg/l, (C) arsenicAs (V) 50 mg/l, and (D) arsenicAs and erythromycin-treated 50 mg/l.

#### 4. CONCLUSION

The present study reported the influence of As in the development of AR in the native bacterium. The isolate PS-6 showed highest As resistance up to 1,000 mg/l and it was identified as *B. licheniformis* using 16S RNA sequencing. Screening of *B. licheniformis* in the presence of As (III) and As (V) against different antibiotics showed resistance toward ampicillin, methicillin, and erythromycin. The growth curve of *B. licheniformis* treated with As (V) with erythromycin showed tolerance of erythromycin by As. The protrusion of the cell wall and pleomorphic modification exhibited by *B. licheniformis* during FESEM analysis acts as a key indicative response for AR. From this study, As was identified as a precursor for the development of AR in the bacterium. Hence, *B. licheniformis* can be considered as an indicator to access the AR in the environment influenced by As. Further studies on the influence of As in AR genes are required for better understanding.

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#### 6. CONFLICT OF INTEREST

The authors declare that they do not have any conflicts of interest.

#### 7. FINANCIAL SUPPORT

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#### 8. CONTRIBUTIONS

Subbiah Kavitha designed the concept and supervised the work. Dhanasekaran Padmanabhan and Zerubabel Stephen performed/carrried out all the research including the data analysis and wrote the manuscript. Somanathan Karthiga Reshmi participated in the revision/reviewed and editing of the manuscript for the final content. All authors read and approved the final manuscript.

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**SUPPLEMENTARY TABLE****Table S1:** Properties of Pichavaram soil sediments.

S.No	Parameter	Results
1.	Appearance	Fine powder
2.	Color	Brownish Gray
3.	pH	6.7
4.	Al	3.83 %
5.	As	BDL
6.	Ca	8.05%
7.	Fe	24.35%
8.	Mg	0.91%
9.	Si	17.84%
10.	Organic carbon	0.72%
11.	Total nitrogen	0.02%
12.	Total phosphorus	0.005%
13.	Total potassium	0.013%

pH = Percentage of hydrogen ion concentration; Al = Aluminum; As =Arsenic; Ca =Calcium; Fe = Iron; Mg = Magnesium; Si = Silica; BDL = Below detectable limit.