Incidence of Arsenic Resistant Bacteria Isolated From a Sewage Treatment Plant.

Anyawu, C.U.* and Ugwu, C.E.
Department of Microbiology University of Nigeria Nsukka, Nigeria.
chukwudi.anyanwu@unn.edu.ng

Abstract--This study was carried out to assess the incidence of arsenic resistant bacteria from a sewage treatment plant receiving various waste effluents from a university community. Twelve arsenic resistant bacteria were isolated from the oxidation ponds and sewage sludge by growing them on nutrient agar medium amended with high concentrations of arsenic. The isolates were studied based on their cultural, morphological and biochemical characteristics and were identified as strains of Pseudomonas aeruginosa (3), Bacillus spp (3), Flavobacterium spp (2), Escherichia coli (2), Klebsiella sp (1) and Staphylococcus aureus (1). Further study of the twelve isolates showed that six of them exhibited resistance to arsenic concentration of 200 ppm and above. Co-resistance of the six isolates to some heavy metals, namely, Cd²⁺, Cu²⁺, and Cr⁶⁺ was studied and all the six isolates showed high resistance to the heavy metals with minimum inhibitory concentration (MIC) for the heavy metals ranging from 100 to 400 ppm. The bacterial isolates obtained in the present study could be successfully exploited biotechnologically for the bioremediation of arsenic and heavy metal contaminated ecosystem.

Index Term--Arsenic, heavy metal, resistance, bacteria, sewage.

I. INTRODUCTION
Metal contaminants are commonly found in soils, sediments, and water. Metal pollutants can be produced through industrial processes such as mining, refining, and electroplating. Metals are not biodegradable but can be transformed through sorption, methylation, and complexation, and changes in valence state [1]. These transformations affect the mobility and bioavailability of metals. At low concentrations, metals can serve as important components in life processes, often serving important functions in enzyme productivity. However, above certain threshold concentrations, metals can become toxic to many species. Fortunately, microorganisms can affect the reactivity and mobility of metals. Microorganisms that affect the reactivity and mobility of metals can be used to detoxify some metals and prevent further metal contamination.

Arsenic is one of the naturally occurring elements in the earth’s crust and a notable element that is present in trace amounts in a healthy human body. The element is widely distributed in nature resulting from weathering, fire, dissolution, volcanic activity and anthropogenic input [9]. High levels of arsenic, however, can cause very grave health problems. In its inorganic form, it is considerably more toxic than its organic derivatives. Arsenic is used in pesticides, herbicides, wood preservatives and dyestuffs as well as production of arsenic-containing wastes during smelting and mining operations[22] Arsenic’s toxic and medicinal properties have been appreciated for more than two millennia[8].

A major concern for arsenic-enriched environments is the potential for mobilization and transport of this toxic element to ground water and drinking water supplies. Its two soluble inorganic forms, arsenite (+3) and arsenate (+5), entering drinking water from natural sources, have caused poisoning in Taiwan, Chile, Argentina, Bangladesh and West Bengal, and arsenicosis (arsenic poisoning) has been detected in people from Cambodia, Vietnam, Nepal, China, Inner Mongolia, Bolivia and Mexico [17, 21]. In addition, arsenic contamination due to anthropogenic activity (e.g. mining) is increasing in importance in parts of the USA, Canada, Australia, Argentina and Mexico [26]. Human population can be exposed to arsenic in a number of ways which include ingestion of arsenic in drinking water or food.

Although arsenic is toxic to most organisms, some prokaryotes have evolved mechanisms to gain energy by either oxidising or reducing it [18,28].

Prokaryotic arsenic metabolism has been detected in hydrothermal and temperate environments and has been shown to be involved in the redox cycling of arsenic [12,19]. The arsenite-oxidising bacteria isolated so far are phylogenetically diverse. The oxidation of arsenite may yield useable energy or may merely form part of a detoxification process [9]. Research is also discovering anaerobic bacteria species that can achieve respiratory reduction of As (V) to As (III) [31].

Today’s industrial world has contaminated our soil, sediment, and water sources with hazardous materials. Metal waste is often a result of industrial activities, such as mining, refining, and electroplating. Mercury, arsenic, lead, and chromium are often prevalent at highly contaminated sites [1]. This fact holds significant challenges for industries because these metals are difficult to remove. Therefore researchers and industries are researching on metals that undergo methylation, complexation, or changes in valence state. These are noteworthy processes because they aid the mobility and bioavailability of metals [16]. There is a large interest in microorganisms that can facilitate the transformation and the removal of the metal contaminant.
This study aimed to isolate and identify arsenic resistant bacteria from a sewage treatment plant and also study their potential for co-resistance to some heavy metals.

II. MATERIALS AND METHODS

Sample collection

Wastewater samples and sludge were collected from the sewage treatment plant in the University of Nigeria, Nsukka in Southeast Nigeria. The plant received a mixture of domestic sewage and wastes from several homes, laboratories and establishments. Sewage sludge from the sludge tank and water samples from the oxidation ponds were aseptically collected in sterile screw-capped bottles and transported immediately to the laboratory for analysis.

Isolation and identification of arsenic resistant bacteria

For the selective isolation of arsenic resistant bacteria, ten-fold serial dilutions of the sewage sludge and sewage water samples were prepared. An aliquot (0.1 ml) of the diluted samples was spread-inoculated on sterile nutrient agar plates amended with 25 and 50 ppm of arsenic. Control plates were set up without arsenic. The plates were incubated at 37°C for 48 h. After the incubation period, the plates were observed for growth on the media. The isolated and distinct colonies on the media were subcultured repeatedly on the same media for purification. The purified isolates were put into nutrient agar slants for storage at refrigeration temperature (4°C). The isolates were identified on the basis of their morphology and biochemical characteristics following the schemes of [6] and [7].

Growth of the isolates on different concentrations of arsenic

Test tubes of nutrient broth were amended with different concentrations of arsenic, namely, 40, 60, 80, 100 and 150 ppm, respectively. Each isolate was inoculated into the different concentrations of arsenic and the tubes were incubated at 37°C for 48 h after which growth of each isolate was determined spectrophotometrically by measuring the optical density (OD) at 600 nm. After this, the isolates that exhibited greater amount of growth at 100 ppm arsenic and above were selected for further study. Growth was quantified relative to growth of controls containing no arsenic.

Determination of co-resistance to some heavy metals

The selected isolates that exhibited resistance to 100 ppm or more of arsenic were tested for their resistance to some heavy metals, namely, Cd\(^2+\), Cu\(^2+\) and Cr\(^6+\). Each selected isolate was freshly inoculated into tubes of nutrient broth containing 100 ppm concentration of each heavy metal as earlier described for arsenic. Growth was determined spectrophotometrically at 600 nm as earlier described for arsenic.

Determination of minimum inhibitory concentration (MIC) of the heavy metals

The MIC of the heavy metal resistant bacterial isolates grown on heavy metal amended media was determined by gradually increasing the concentration of the heavy metal by 20 ppm each time on nutrient agar plates until the isolate failed to show colony growth on the plate. The starting concentration used was 80 ppm. The culture growing on the last concentration was transferred to the next higher concentration by streaking on the plate medium. The MIC was noted when the isolate failed to show growth on the plates even after seven days of incubation [23]. All experimental set-ups were prepared in duplicate.

III. RESULTS

Samples of wastewater and sludge collected at different points in the sewage treatment plant contained arsenic resistant bacteria. When the sewage water and sludge samples were cultured on nutrient agar amended with arsenic, twelve isolates were found to grow in nutrient agar plates with 50 ppm arsenic (Table I). Of the twelve isolates, three were obtained for each of *Pseudomonas aeruginosa* and *Bacillus* spp., two isolates for each of *Flavobacterium* spp. and *Escherichia coli*, and one isolate for each of *Klebsiella* sp. and *Staphylococcus aureus*. Out of the twelve isolates, seven were obtained from the sludge samples while five isolates were obtained from the sewage water (Table I). The isolates were identified based on their morphological and biochemical characteristics.

When the twelve isolates were grown further at higher concentrations of arsenic, six isolates were found to grow effectively at arsenic concentration of 200 ppm or more. Results of the growth responses of the six selected strains of bacteria on different concentrations of arsenic are as shown in Figure 1. The growth response, which was measured spectrophotometrically at 600 nm, was observed to be decreasing with increase in the concentration of arsenic. Of the six isolates, *Pseudomonas aeruginosa* (PAR3) was observed to show the highest level of growth at 200 ppm arsenic while *Klebsiella* sp (KLB1) exhibited the least amount of growth at the same concentration (Fig. 1).

All the six selected isolates that showed resistance to higher concentrations of arsenic also showed resistance to the heavy metals studied, with MIC for the heavy metals ranging from 100 ppm to 400 ppm (Table II). Thus, all the arsenic resistant bacteria tested showed multiple resistances to the heavy metals.

The resistance test indicated that among the three experimental heavy metals, chromium at 400 ppm completely inhibited the growth of *Pseudomonas aeruginosa* while at 100 ppm copper, the growth of *Flavobacterium* sp. and *Klebsiella* sp. were completely inhibited, hence making these as the MIC of these metals for the isolates. In all the isolates, a decrease in growth (measured in terms of optical density) was observed upon increasing concentration of heavy metals at any given time interval compared to the control without metal amendment (data not shown). The lower optical density values indicated toxic effect of the heavy metals on the growth of microorganisms. In the case of *Pseudomonas aeruginosa*, the growth increased steadily over the entire incubation period. However, the reduction in the growth in the presence of increased concentration of the metals tested in the study was evident throughout the experimental period compared with the control.
IV. DISCUSSION

This study resulted in the isolation and purification of 12 bacterial isolates from sewage wastewater and sludge from a sewage treatment plant. These isolates have high ability to resist and tolerate arsenic. Six of the twelve isolates were screened for their metal resistance in nutrient broth medium containing different metal salt concentrations of each of Cu²⁺, Cd²⁺ and Cr³⁺, respectively, which ranged between 80 and 400 ppm in order to determine bacterial isolates capable of growing and resisting high levels of metal toxicity. The MIC of each of the six bacterial isolates for each of the tested heavy metals was thus investigated using nutrient agar plate assay and presented in the results.

Arsenic compounds have been widely used as biocides both for research purposes and in agriculture, industry, and medicine because of their toxicity to microorganisms, plants, insects, and mammals. They are also used as selective enzyme inhibitors in biochemical research. Reports had indicated that low concentration of metals is essential for the growth, but increasing concentration levels are inhibitory to growth and may even exert lethal effects on the organisms [29, 3]. The concentration of the metal present in the medium influences the response of the organism to that particular metal. When the concentration of the metal was increased above tolerable levels, the initial response was an inhibition of microbial growth and activity. As the metal concentration was increased, death was ensured.

In the present study, it was observed that as the metal concentration in the medium increased, growth of the cells steadily decreased until the MIC concentration, where no growth was observed. In the control set, immediate growth was observed but growth in cultures with lower concentrations of the heavy metal started late with a lag period of up to 6 to 12 hours very prominent. Similar results were reported by other researchers too [5]. The lag in the presence of the heavy metal may be due to the stress caused by high concentration of the metal ion which affects the effectiveness of the membrane in transporting material needed for normal growth of the organism. Due to prolonged lag phase there is delay in log phase. It is considered that heavy metals inhibit enzymatic reactions through either their complexing with substrate or blocking the functional groups of enzymes or reacting with complex enzyme-substrate [27].

Out of the 12 isolates obtained from different plates of arsenic-amended media, it was observed that almost 67% of the strains were gram negative at higher metal concentration and the remaining were gram positive. Common gram positive bacteria at higher concentration belonged to genus Bacillus and Staphylococcus. At lower concentrations, morphological diversity was more as compared to at higher metal concentrations. The predominance of gram-negative bacteria at higher concentration of metal is probably due to their higher level of intrinsic arsenic resistance than majority of the gram-positive bacteria. The basis of this difference might be due to the differences in the chemical composition of cell wall of gram-negative bacteria and gram-positive bacteria [4].

In the present study, toxicity of the heavy metals was concentration and time dependent for each group of sewage organisms. No specific pattern of toxicity was observed against these diverse microbial groups. However, in general, the microbial isolates were more resistant to arsenic followed by chromium, cadmium and then least resistant to copper. Inhibitory or lethal concentration of a metal is dependent on various factors which include both the metal and the organism. The susceptibility of different species to heavy metals varies enormously [7]. Sensitivity and susceptibility of an organism to heavy metal attack varies considerably among different species and also between populations of one single strain depending on physiological state of the culture [14, 15].

Several arsenic resistant bacteria had been isolated from different sources. Seventeen morphologically distinct arsenic resistant heterotrophic bacteria were isolated by Anderson and Cook [2].These bacteria included members of the genera Aeromonas, Bacillus, Pseudomonas, Escherichia and Acinetobacter, respectively. Bacillus licheniformis, Bacillus polymyxa, Pseudomonas fluorescens and Listeria murrayi had been isolated as arsenic resistant bacteria [27]. All the bacterial strains were able to tolerate more than 100 ppm arsenic. Members of culturable arsenic (v) resistant bacteria had been isolated from which some were capable to tolerate very high (100 mM) level of arsenate [13]. In addition, Zelibor [30] isolated arsenic (v) resistant bacteria from well water samples which tolerated up to 2,000 µg of As (v) per ml.

Microbial exposure to heavy metals selects and maintains microbial variants able to tolerate the harmful effects of metals. Varied and efficient metal resistance mechanisms have been identified in diverse species of bacteria. Bacteria that showed tolerance to chromium up to 500 µg/ml were isolated by Rajbanshi [20]. All the isolates used in this study showed multiple resistances to the heavy metals. The bacterial resistance to heavy metals is attributable to a variety of detoxifying mechanisms developed by resistant microorganisms such as complexation by exopolysaccharides, binding with bacterial cell envelopes, metal reduction, metal efflux, etc. These mechanisms are sometimes encoded in plasmid genes facilitating the transfer of toxic metal resistance from one cell to another [25]. Several bacterial isolates including Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis and Staphylococcus sp, resistant to heavy metals and antibiotics were isolated [10]. Similarly, Sharma et al. [24] isolated highly cadmium resistant Klebsiella that was found to precipitate significant amount of cadmium sulphide.

Since heavy metals are all similar in their toxic mechanisms, multiple tolerances are common phenomena among heavy metal resistant bacteria [20]. Heavy metal resistant bacteria could be potential agents for bioremediation of heavy metal pollution. Therefore, the bacterial isolates obtained in the present study could be successfully exploited biotechnologically for the bioremediation of arsenic and heavy metal contaminated ecosystem.

REFERENCES


Table 1
Bacterial isolates resistant to arsenic

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>Source of isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Pseudomonas aeruginosa</em></td>
<td>PAR1</td>
</tr>
<tr>
<td>2. <em>Pseudomonas aeruginosa</em></td>
<td>PAR2</td>
</tr>
<tr>
<td>3. <em>Pseudomonas aeruginosa</em></td>
<td>PAR3</td>
</tr>
<tr>
<td>4. Bacillus sp.</td>
<td>BAC1</td>
</tr>
<tr>
<td>5. Bacillus sp.</td>
<td>BAC2</td>
</tr>
<tr>
<td>6. Bacillus sp.</td>
<td>BAC3</td>
</tr>
<tr>
<td>7. Flavobacterium sp.</td>
<td>FLA1</td>
</tr>
<tr>
<td>8. Flavobacterium sp.</td>
<td>FLA2</td>
</tr>
<tr>
<td>9. Escherichia coli</td>
<td>ECO1</td>
</tr>
<tr>
<td>10. Escherichia coli</td>
<td>ECO2</td>
</tr>
<tr>
<td>11. Klebsiella sp.</td>
<td>KLB1</td>
</tr>
<tr>
<td>12. Stapylococcus aureus</td>
<td>STA1</td>
</tr>
</tbody>
</table>
### Table II: MIC of arsenic and heavy metals for the bacterial isolates

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>MIC (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cu$^{2+}$</td>
</tr>
<tr>
<td>PAR2.</td>
<td>240</td>
</tr>
<tr>
<td>PAR3.</td>
<td>200</td>
</tr>
<tr>
<td>BAC6.</td>
<td>100</td>
</tr>
<tr>
<td>FLA1</td>
<td>240</td>
</tr>
<tr>
<td>ECO1.</td>
<td>200</td>
</tr>
<tr>
<td>KLB1</td>
<td>280</td>
</tr>
</tbody>
</table>

![Graph showing effect of different concentrations of arsenic on the growth of isolates.]

**Fig 1**: Effect of different concentrations of arsenic on the growth of the isolates.