Tolerance to and removal of arsenic (III) by bacteria of the genus *Bacillus* sp. isolated in Chihuahua, Mexico

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ABSTRACT: *Arsenic contamination threatens aquatic environments and motivates the selection of microorganisms capable of tolerating and removing arsenic. Three Bacillus sp. isolates (ANAs215002, ANPb214001, and ANCd115006) were compared for observable growth on nutrient agar containing 0–200 mg/L As(III), growth response in liquid medium containing 100 mg/L arsenic, and changes in aqueous total arsenic over 48 h. The maximum tested concentrations supporting observable growth were 50, 75, and 100 mg/L, respectively. At 48 h, apparent aqueous arsenic removal reached 19.38%, 31.53%, and 51.52%, respectively. ANCd115006 combined the highest apparent tolerance and greatest arsenic removal, supporting its prioritization for mechanistic follow-up. Because arsenic speciation and a mass balance were not measured, the observed decrease in the analyzed fraction cannot be assigned to biosorption, bioaccumulation, precipitation, or redox transformation.*

KEYWORDS – *Arsenic, Bacillus, arsenite, Bioremediation.*

I. INTRODUCTION

Arsenic (As) is a highly toxic metalloid whose presence in aquatic ecosystems represents a global environmental and public health concern. Arsenic contamination may originate from natural processes, such as mineral weathering and interactions between rocks and groundwater, as well as anthropogenic activities associated with mining, metallurgy, agriculture, and various industrial processes. In aquatic systems, arsenic occurs primarily in two oxidation states: arsenite [As(III)] and arsenate [As(V)], whose distribution depends on pH and redox potential. As(III) generally exhibits greater toxicity and mobility than As(V), posing a significant challenge for its removal from contaminated environments [1,2]. Conventional technologies for treating arsenic-contaminated water include coagulation, chemical precipitation, adsorption, ion exchange, and membrane filtration. However, these technologies may present limitations related to operational costs, secondary waste generation, and treatment efficiency under specific physicochemical conditions. Therefore, bioremediation using arsenic-resistant microorganisms has gained increasing attention as a potentially complementary alternative to conventional treatment methods [2,3].

Various microorganisms have developed physiological and molecular mechanisms that enable them to survive in arsenic-contaminated environments. Reported mechanisms include cellular transport and efflux systems, oxidation of As(III) to As(V), reduction of As(V) to As(III), biosorption onto cell surface components, and intracellular accumulation [3,4]. The biological oxidation of As(III) to As(V) is particularly important because it may facilitate subsequent immobilization through adsorption or

coprecipitation processes. However, bacterial resistance to arsenic does not necessarily imply removal capacity, as certain resistance mechanisms promote cellular survival without decreasing the metalloid concentration in the surrounding medium [4]. In this context, bacteria belonging to the genus *Bacillus* have attracted considerable interest due to their physiological diversity, adaptability, and potential to interact with inorganic contaminants through mechanisms such as biosorption, bioaccumulation, and bioprecipitation [5]. Flores et al. [6] investigated *Bacillus paralicheniformis* ZAP17 and *Bacillus altitudinis* ZAP62 strains isolated from geothermal environments in Araró, Mexico, and identified the arsenic resistance operons *arsRBC* and *arsRBCDA*, whose expression in the presence of As(III) provided molecular evidence of mechanisms associated with resistance to this metalloid. These findings demonstrate that bacterial responses to arsenic depend on strain-specific characteristics and highlight the need to experimentally evaluate both tolerance and removal capacity.

Molecular identification based on 16S ribosomal RNA (16S rRNA) gene sequence analysis is an essential tool for the taxonomic and phylogenetic characterization of environmental bacteria. Soto-Padilla et al. [7] and Cruz-Soto et al. [8] previously reported the molecular identification of the bacterial isolates investigated in the present study, providing a foundation for their subsequent physiological and biotechnological evaluation. Therefore, the objective of this study was to evaluate As(III) tolerance and arsenic removal capacity in three bacterial isolates belonging to the genus *Bacillus*, identified as ANAs215002, ANPb214001, and ANCd115006, through experimental assays conducted in synthetic systems.

II. MATERIALS AND METHODS

II.1. Strain activation

The bacterial strains were activated on nutrient agar according to the optimal growth conditions reported for each strain by Soto-Padilla et al. and Cruz-Soto et al. [7,8]. The plates were incubated at 37 °C for 48 h.

II.2.Minimum inhibitory concentration (MIC)

Arsenite tolerance was evaluated by determining the minimum inhibitory concentration (MIC). The three *Bacillus* strains were exposed to increasing concentrations of As(III) (25, 50, 75, 100, 150, y 200 mg/L), using arsenic trioxide (As_2O_3) as the arsenite source. Petri dishes were prepared in triplicate for each concentration and bacterial strain; in addition, uninoculated control was used for all concentrations. The Petri dishes were incubated at 37°C and growth was observed at 72 h. The MIC was defined as the lowest concentration at which no detectable bacterial growth was observed [7].

II.3. Growth kinetics

The bacterial strains were inoculated at 10% (v/v) using a pre-inoculum and incubated in Erlenmeyer flasks. For the As(III) growth kinetics assay, Nutrient Broth (DIFCO) supplemented with arsenic trioxide (As_2O_3) to a final As(III) concentration of 100 mg/L was used. The cultures were incubated at 37 °C and 200 rpm. Microbial growth was quantified by turbidimetry using 3 mL aliquots collected at different time intervals (0, 4, 24, and 48 h). The optical density of each sample was measured at 600 nm using a UV-Vis spectrophotometer (Lambda 2) [13]. All assays were performed in triplicate.

II.4. As(III) remotion kinetics

As(III) remotion was evaluated using Nutrient Broth supplemented with arsenic trioxide (As_2O_3) to a final As(III) concentration of 100 mg/L. The cultures were adjusted to an initial optical density (OD_{600}) of 0.8 and incubated at 37 °C and 200 rpm for 48 h. As(III) remotion was evaluated by collecting 5 mL aliquots of the cell suspension at different time intervals (0, 4, 24, and 48 h). The samples were centrifuged at 3000 rpm for 15 min, and the supernatant was recovered to determine the residual As(III). The analyzed fraction underwent acid digestion with HCl and HNO_3 and was quantified by the atomic absorption (Perkin-Elmer, Canada) [7]. All assays were performed in triplicate.

III. RESULTS AND DISCUSSION

The three isolates of the genus *Bacillus* exhibited differences in their tolerance to As(III), demonstrating a response that depended on both the specific isolate and the concentration of the metalloid (Fig. 1). Strain ANAs215002 showed the highest tolerance, maintaining detectable growth throughout the entire range tested from 25 to 200

mg/L of As(III). Meanwhile, strain ANCd115006 exhibited growth up to 100 mg/L, whereas strain ANPb214001 showed growth up to 75 mg/L. The lack of growth for ANCd115006 at concentrations of 150 mg/L and above, and for ANPb214001 at 100 mg/L and above, allowed for the determination of apparent minimum inhibitory concentrations (MICs) of 150 mg/L and 100 mg/L, respectively. In contrast, it was not possible to determine an MIC for ANAs215002, as it continued to grow at the highest concentration tested. These results highlight variations in the response to As(III) among isolates belonging to the same bacterial genus.

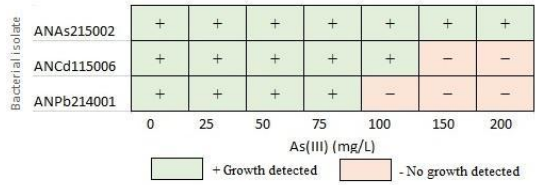


Fig. 1. As(III) tolerance of three Bacillus sp. isolates at different arsenite concentrations. Green cells indicate detectable bacterial growth (+), whereas pink cells indicate no detectable growth (−). Isolate ANAs215002 exhibited growth at concentrations up to 200 mg/L, while ANCd115006 and ANPb214001 showed apparent minimum inhibitory concentrations (MICs) of 150 and 100 mg/L, respectively.

The observed tolerance can be compared with the findings of a study published in *Soil Systems* [9], which evaluated 45 bacterial isolates obtained from contaminated soils. Among these, the *Bacillus* sp. strains BS5 and BS6 exhibited arsenite MICs exceeding 4 mM, equivalent to more than 299.7 mg/L of As(III). Although these values exceed the range tested in the present study, they provide evidence that bacteria of the genus *Bacillus* can continue to grow when exposed to arsenite concentrations in the range of several hundred milligrams per liter. Furthermore, Ordóñez et al. [10] reported that *Bacillus subtilis* exhibits resistance to 2.5 mM As(III), which is approximately equivalent to 187.3 mg/L. This value falls within the experimental range evaluated in the present study and is particularly relevant for contextualizing the tolerance observed in ANAs215002.

The three *Bacillus* strains exhibited different growth patterns during 48 h of incubation in the presence of 100 mg/L As(III), indicating variations in their physiological responses under the experimental conditions evaluated (Fig. 2). Strain ANAs215002 showed a sustained increase in optical density, reaching approximately 1.30 and 1.98 at 24 and 48 h, respectively. In contrast, ANCd115006 exhibited a higher optical density during the first 24 h (OD_{600} ≈ 1.60), followed by a slight decrease to approximately 1.50 at 48 h. Meanwhile, ANPb214001 exhibited the lowest optical density values throughout the experimental period, reaching an OD_{600} of approximately 0.70 at the end of the assay. These differences indicate that the isolates exhibited distinct growth responses to the same As(III) concentration.

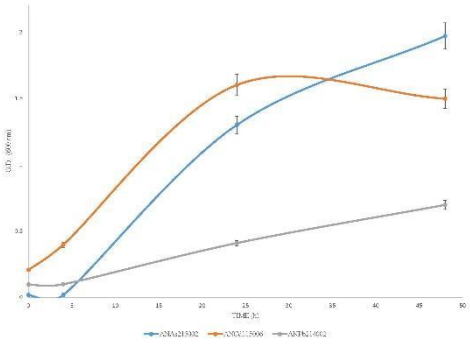


Fig. 2. Growth profiles of three Bacillus sp. isolates (ANAs215002, ANCd115006, and ANPb214001) during 48 h of incubation in the presence of 100 mg/L As(III).

These findings are consistent with previous studies demonstrating differences in the responses of arsenic-resistant bacteria during exposure to this metalloid. Roy et al. [11] evaluated the growth of two arsenic-resistant bacterial isolates, designated PF14 and KG1D, in media supplemented with As(III) and As(V). The authors observed that As(III) exposure altered growth parameters compared with arsenic-free controls, resulting in lower growth rates and longer generation times. These findings demonstrate that the ability to survive in the presence of arsenic does not necessarily imply maintaining growth comparable to that observed in the absence of the contaminant. In the present study,

the differences in optical density among ANAs215002, ANCd115006, and ANPb214001 suggest distinct physiological responses.

The growth capacity of the isolates can also be contextualized by the findings of Kabiraj et al. [12], who characterized seven Bacillus species isolated from arsenic-contaminated groundwater in India. The authors reported that these microorganisms maintained growth in the presence of arsenite salt concentrations exceeding 0.65 g/L and exhibited resistance to other metals. These findings support the ability of certain bacteria belonging to the genus Bacillus to grow in environments containing elevated arsenic concentrations.

The differences observed among the strains may be associated with specific arsenic resistance mechanisms. William and Magpantay [13] described that bacterial survival in the presence of this metalloid may involve transport and cellular efflux systems, regulation of ars operons, and transformation processes involving inorganic arsenic species. These mechanisms contribute to reducing intracellular toxicity and may help maintain physiological activity during arsenic exposure.

The three *Bacillus* strains exhibited differences in their ability to reduce arsenic concentrations in the aqueous phase during 48 h of incubation (Fig. 3). ANCd115006 showed the highest removal efficiency, reaching 35.72% at 4 h, 45.41% at 24 h, and 51.52% at 48 h. In contrast, ANAs215002 exhibited removal percentages of 14.18%, 16.02%, and 19.38% at the same sampling times, whereas ANPb214001 showed a progressive increase, with removal values of 2.34%, 17.40%, and 31.53%, respectively. These results demonstrate that arsenic removal capacity was isolate-specific and depended on incubation time.

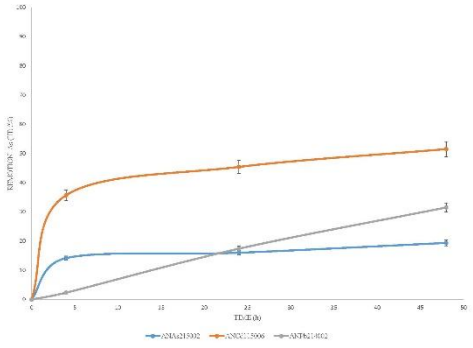


Fig. 3. Time-dependent arsenic removal by three Bacillus sp. isolates (ANAs215002, ANCd115006, and ANPb214001) during 48 h of incubation in the presence of 100 mg/L As(III). Arsenic removal was expressed as the percentage decrease in aqueous arsenic concentration relative to the initial concentration.

The removal patterns observed for ANCd115006 and ANAs215002 were characterized by a relatively rapid decrease in aqueous arsenic concentrations during the first 4 h, followed by smaller increases in removal efficiency. This trend may be associated with an initial interaction between arsenic and bacterial cell surface components, although the present results do not establish whether biosorption or another retention mechanism was involved. Roy et al. [11] investigated two arsenic-resistant bacterial isolates, designated PF14 and KG1D, and demonstrated that arsenic removal may involve both biosorption onto bacterial biomass and intracellular bioaccumulation. Furthermore, the authors reported differences between isolates depending on pH conditions, indicating that arsenic–bacteria interactions are influenced by the specific characteristics of each microorganism and the exposure medium.

In contrast, ANPb214001 exhibited a different removal pattern, characterized by a gradual increase in arsenic removal throughout the 48 h incubation period. While only 2.34% removal was recorded at 4 h, removal increased to 17.40% and 31.53% at 24 and 48 h, respectively. This response suggests that the decrease in aqueous arsenic concentration occurred more progressively than in the other isolates. Previous studies on arsenic-resistant bacteria have demonstrated that arsenic removal may involve multiple processes acting simultaneously or contributing differently throughout incubation. In particular, a study involving *Pseudomonas* sp. SMS11 demonstrated the involvement of As(III) oxidation, biosorption, and bioaccumulation through the evaluation of dissolved, cell surface-associated, and intracellular arsenic fractions [14]. These findings highlight the importance of distinguishing among the processes responsible for arsenic removal from aqueous media. However, these mechanisms cannot be attributed to ANPb214001 without additional experimental evidence.

The differences among the isolates may also be associated with their physiological characteristics and their ability to interact with

arsenic. William and Magpantay [13] described several bacterial arsenic resistance and transformation mechanisms, including cellular efflux systems, As(III) oxidation, As(V) reduction, and immobilization processes. However, bacterial resistance does not necessarily result in greater arsenic removal efficiency, since some resistance mechanisms promote cellular survival without reducing arsenic concentrations in the surrounding medium. This distinction is particularly relevant to the present study, as ANAs215002 exhibited the highest As(III) tolerance and reached the highest optical density at 48 h but showed the lowest final arsenic removal percentage (19.38%). In contrast, ANCd115006, which exhibited lower arsenic tolerance, achieved the highest removal efficiency (51.52%). Therefore, these findings suggest that bacterial tolerance and growth are not sufficient indicators for predicting arsenic removal capacity.

The influence of microorganism-specific characteristics on arsenic removal has also been documented in recent investigations. Khandelwal et al. [15] evaluated As(III) biosorption using immobilized bacterial biomass of Acinetobacter sp. Sp2b and reported that arsenic retention depended on several factors, including contact time, pH, initial arsenic concentration, and biosorbent dosage. Although their experimental system differs from that used in the present study, their findings support the importance of operational conditions and biomass properties in explaining differences in arsenic removal efficiency.

Overall, ANCd115006 exhibited the highest capacity to decrease arsenic concentrations in the aqueous phase, followed by ANPb214001 and ANAs215002. The differences in their time-dependent removal patterns suggest that the processes contributing to arsenic removal may vary among the isolates. Nevertheless, further investigations involving As(III)/As(V) speciation, quantification of biomass-associated arsenic, and analysis of extracellular and intracellular arsenic fractions are required to identify the mechanisms involved. These findings provide preliminary evidence of the potential of these *Bacillus* isolates for further investigations aimed at developing bioremediation strategies for arsenic-contaminated aquatic systems.

IV. Conclusion

The differences observed among the isolates demonstrate that higher As(III) tolerance and bacterial growth do not necessarily correspond to greater arsenic removal efficiency. This finding highlights the importance of evaluating tolerance, growth, and removal capacity as complementary but distinct characteristics when selecting bacterial strains for environmental applications. Among the isolates evaluated, ANCd115006 emerged as the most promising candidate for further investigation due to its comparatively high arsenic removal efficiency. Nevertheless, additional studies involving arsenic speciation, biomass-associated arsenic quantification, and the identification of extracellular and intracellular retention mechanisms are required to determine the processes responsible for arsenic removal. Overall, these findings contribute to the understanding of strain-specific responses to arsenite exposure and provide a basis for the selection of bacterial isolates with potential applications in the development of sustainable bioremediation strategies for arsenic-contaminated aquatic environments.

Acknowledgements

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