Research Articles

Arsenic-Contaminated Soils

Genetically Modified *Pseudomonas* spp. and Their Arsenic-Phytoremediation Potential

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Abstract. Sorghum was inoculated with *Pseudomonas* bacteria, including strains harboring an As-resistance plasmid, pBS3031, to enhance As-extraction by the plants. *Pseudomonas* strains (*P. fluorescens* 38a, *P. putida* 53a, and *P. aureofaciens* BS1393) were chosen because they are antagonistic to a wide range of phytopathogenic fungi and bacteria, and they can stimulate plant growth. The resistance of natural rhizospheric pseudomonads to sodium arsenite was assessed. Genetically modified *Pseudomonas* strains resistant to As(III)/As(V) were obtained via conjugation or transformation. The effects of the strains on the growth of sorghum on sodium-arsenite-containing soils were assessed.
The conclusions from this study are: (1) It is possible to increase the survivability of sorghum growing in sodium-arsenite-containing soil by using rhizosphere pseudomonads. (2) The presence of pBS3031 offers the strains a certain selective advantage in arsenite-contaminated soil. (3) The presence of pBS3031 impairs plant growth, due to the As-resistance mechanism determined by this plasmid: the transformation of the less toxic arsenate into the more toxic, plant-root-available arsenite by arsenate reductase and the active removal of arsenite from bacterial cells. (4) Such a mechanism makes it possible to develop a bacteria-assisted phytoremediation technology for the cleanup of As-contaminated soils and is the only possible way of removing the soil-sorbed arsenates from the environment.

Keywords: Arsenic-contaminated soils; As-resistance plasmid; phytoremediation; *Pseudomonas*; rhizosphere

Abbreviations: **CFU:** Colony-Forming Unit; **LB:** Luria-Bertoni; **MIC:** Minimum Inhibitory Concentration

Introduction

The Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on Their Destruction (Paris 1993) stipulates that the vesicant chemical-warfare agents mustard and Lewisite be destroyed first of all. Lewisite [dichloro(2-chlorovinyl)arsine; Cl-CH=CH-$AsCl_2$] is an arsenic (As)-containing agent. Its industrial detoxification gives rise to sodium arsenite, an alkaline-hydrolysis product of Lewisite. Thus, alongside the mining and metallurgical industries and the wide, uncontrolled, heavy application of As-containing pesticides up to the mid-20th century, the industrial destruction of Lewisite may soon become a major potential source of As on the planet.

Soils often contain high concentrations of various natural and man-made compounds of As. As is regarded to be moderately phytotoxic because, like Se, Cd, Zn, Mn, and Cr ions, As ions are inhibitory at solution concentrations of 1 to 100 mg/L. (In contrast, the highly phytotoxic Hg, Be, Sn, Ag, and Pb adversely affect test organisms when present at solution concentrations of up to 1 mg/L.) [1]. Arsenites [As(III)] and arsenates [As(V)] are most important for the interaction with soil biota. Arsenites are powerful inhibitors of sulfhydryl groups. They inactivate microbial enzymes and attack plant-cell membranes, thus suppressing root function on contact with roots or causing a rapid necrosis on contact with leaves. Arsenates do not damage membranes, because they do not react with sulfhydryl groups; however, arsenates do affect phosphorylation in mitochondria [2].

Most As present in soil is sorbed by soil colloids, mainly by the clay components Fe oxide and Al oxide. Sorption of As by clay depends on the type and pH of soils, the presence and state of Fe and Al, the amount and type of clays in soils, and the presence of organic compounds. The amount of adsorbed As increases with an increase in clay content. The amount of soluble As takes about four months (from one to six) to decrease to a constant value, and the rate of decrease is different for different soils. Initially, the adsorption of arsenicals proceeds fast; then, a long-term transformation of As into low-soluble forms begins [1].

Sorption of As reduces the toxicity of the element. Data available indicate that arsenicals are more toxic in stiff, sandy soils than they are in fine-structured, clay soils. This fact is understandable when it is considered that clay soils have a high sorption capacity toward As [2].

Bacteria take an active part in global As-cycling in the biosphere and detoxify toxic agents in soil by either degrading them or transforming them into less toxic compounds. Microorganisms resistant to As(III)/As(V) occur in various taxonomic groups. The mechanisms of As(III)/As(V) resis-

tance, determined by plasmid as well as chromosomal genes, have been described for *Escherichia coli*, *Alcaligenes faecalis*, *Staphylococcus xylosus*, *S. aureus*, *Acidiphilium multivorum*, *Desulfovibrio* sp., *Desulfomicrobium* sp. and *Pseudomonas* [3–8].

Phytoremediation is a promising environmental technology for soil restoration [9,10]. Sorghum and sunflower can be good candidates to clean up arsenic-contaminated soils [11–13]. Plants can be involved in soil rehabilitation indirectly, via the support of symbiotic and plant-root-associated bacteria. The rhizosphere of plants is rich in root exudates, serving as carbon and nitrogen sources for rhizosphere microorganisms. Fluorescent pseudomonads such as *P. putida*, *P. fluorescens*, *P. aureofaciens*, *P. chlororaphis*, and *P. corrugata* are the predominant gram-negative bacteria in the rhizosphere. Certain strains of these pseudomonads contribute much to the improvement of plant growth and development [14].

The aims of this work were:

- To obtain genetically modified *Pseudomonas* strains resistant to As(III)/As(V).
- To assess the effects caused by the strains on the growth of sorghum on sodium-arsenite-containing soils.

1 Experimental

Bacterial strains and growth conditions. The *Pseudomonas* strains used were the rhizosphere strains *P. fluorescens* 38a, *P. aureofaciens* BS1393 (strain no. V-2188D, All-Russia Collection of Microorganisms), and *P. putida* 53a (strain no. V-1743D, All-Russia Collection of Microorganisms) [15,16]. *E. coli* K802, harboring the conjugative As(III)/As(V)-resistance plasmid pBS3031 [17], was used as the donor to obtain plasmid-containing derivatives of the rhizobacteria. *P. fluorescens* 38a, *P. putida* 53a, and *P. aureofaciens* BS1393 were chosen because they are highly antagonistic to a wide range of phytopathogenic fungi and bacteria, and they can stimulate plant growth.

Bacteria were grown in Luria-Bertoni (LB) broth, on LB agar, or in M9 synthetic medium [18], with glycerol as a carbon and energy source. Growth took place at 30°C either in petri dishes or in Erlenmeyer flasks containing 100 mL of liquid medium and shaken at 150 rpm.

Conjugal transfer of the As-resistance plasmid pBS3031. Twelve-hour donor and recipient cultures (1:2) were spread onto the surface of agar-supplemented LB medium by using a sterile glass spattle. After being incubated for 12 h, the cells were washed off with 0.85% NaCl and were appropriately diluted. The mating-mixture dilutions were plated on selective media containing 500 µg/mL As. The frequency of the conjugal plasmid-transfer was calculated as a ratio of transconjugant number to donor-cell number.

Transformation of the recipient strains with plasmid DNA. Plasmid DNA was isolated as described in [18]. For transformation, a 100-µL portion of overnight culture was grown in 2 mL of LB medium for 2 h. The culture was then transferred aseptically into test tubes and was sedimented by centrifugation (3000 rpm for 15 min) at 0°C. The supernatant liquid was decanted, and 5 mL of 0.1 M $MgCl_2$ was added to the sediment. The sediment was resuspended and centrifuged at 3000 rpm for 10 min. The supernatant was decanted, and 5 mL of 0.15 M $MgCl_2$ was added to the sediment. The sediment was resuspended and kept in ice for 25 min. Next, the sediment was centrifuged for 15 min, the supernatant liquid was decanted, and 1 mL of 0.15 M $MgCl_2$ was added. The sediment was resuspended and kept in ice until used. For transformation, 20 µL of plasmid DNA was added to 200 µL of competent cells. The mixture was left to stand in ice for 1 h and was then heated for 2 min at 42°C. Then, 2 mL of warm LB medium was added and the mixture was left to stand for 5 min. The cells were further cultured for 2 h and were plated on selective medium containing 500 µg/mL As.

Screening for plasmids. Cultures were tested for the presence of plasmid DNA by the conventional method of Eckhardt [19], based on the identification of plasmid DNA by agarose gel electrophoresis with ethidium bromide staining. The electrophoretic conditions were 30 V for 30 min and 100V for 4 h. The 0.8% agarose gels were stained with ethidium bromide for 15 min and were photographed under ultraviolet illumination by using a red filter. The stability of plasmid pBS3031 was tested as reported in [20].

Determination of the minimum inhibitory concentrations. The minimum inhibitory concentrations (MICs) (bacterial-resistance levels) of sodium arsenite and sodium arsenate were determined as described by Carlin et al. [4].

Plant inoculation, soil preparation, and cell enumeration. Sorghum (*Sorghum saccharatum* cv. Pishchevoye-69, The Sorghum Research Institute, Saratov, Russia) seeds were inoculated with suspensions of the *Pseudomonas* strains (density, 10^8 cells/mL), germinated for 24 h at 24°C, and sown into prepared soil. Plants were grown in a growth chamber with a 12-h light period at 25°C and a 12-h dark period at 20°C. There were three experiments, with five replicates per experiment. Uninoculated plants served as controls.

For soil preparation, gray forest soil was passed through a sieve (1 mm) and packed in pots (50 g of soil per pot). To study As toxicity, we used an aqueous solution of sodium arsenite, applied to soil to final As-contents of 75 and 100 mg/kg of soil. Soil was moistened with the solution, and the prepared sorghum seeds were sown in pots, one per pot. Subsequently, the plants were watered with tap water at regular intervals.

For enumeration of bacterial cells on the rhizoplane, the roots of the plants were extensively washed in tap water, rinsed in sterile tap water, placed in 5 mL of sterile physiological saline, and vigorously shaken. The roots were then removed from the saline and were weighed. The resultant suspension was successively diluted 1:10, and 0.1 mL of each dilution was plated on LB agar. After the plates were incubated for 24 h at 30°C, we estimated the colony-forming units (CFUs) for each strain. The CFUs were then estimated per gram of root. The colonies were replicated onto agar-containing selective medium supplemented with sodium arsenite (500 µg/mL), and the number of cells containing plasmid pBS3031 was calculated.

2 Results and Discussion

When the bacteria were grown in LB broth and M9 glycerol-containing medium under nonselective conditions for 144 h, the stability of plasmid pBS3031 in *P. fluorescens* 38a, *P. putida* 53a, and *P. aureofaciens* BS1393 was 100%.

Extension of the growth time to 168 h resulted in the loss of pBS3031 from the pseudomonads. In *P. aureofaciens* BS1393, plasmids were lost by 5% (rich medium) and 1% (deficient medium) of the cells; in *P. fluorescens* 38a, by 13% of the cells; and in *P. putida* 53a, by 5% of the cells. After 264 h of cell cultivation, plasmids were lost by 13% (rich medium) and 3% (deficient medium) of the *P. aureofaciens* BS1393 cells, by 33% of the *P. fluorescens* 38a cells, and by 18% of the *P. putida* 53a cells.

As(III)/As(V)-resistance studies showed that *P. fluorescens* 38a and *P. putida* 53a were As-sensitive, with MICs of 0.23 mM sodium arsenite and 10 mM sodium arsenate. *P. aureofaciens* BS1393 proved to be the most As-resistant, with MICs of 2.5 mM sodium arsenite and 150 mM sodium arsenate. To increase the As resistance in these strains, we performed conjugal transfer of plasmid pBS3031 which was previously found in the clinical isolate *P. aeruginosa* BS3031 [17].

Plasmid pBS3031 is 45 kilobases in size, is conjugative, and is capable of functional expression in *E. coli* and *Pseudomonas* cells, providing for their resistance to sodium arsenite at 500–2000 µg/mL. PCR analysis found that pBS3031 contained a gene, *arsC*, coding for arsenate reductase. Thus, the mechanism of resistance to As(III)/As(V) was determined to include the reduction of AsO_4^{3-} to AsO_2^- and the active removal of As(III) from the cell.

We performed conjugal transfer (frequency, 10^{-8}) of pBS3031 from the donor strain, *E. coli* K802, to the recipient strains *P. fluorescens* 38a and *P. aureofaciens* BS1393. No plasmid transfer occurred during mating of strain K802 with *P. putida* 53a; therefore, we produced a plasmid-containing derivative of this strain by transformation of plasmid DNA (frequency, 10^{-4}). The presence of pBS3031 in the transformants and transconjugates was confirmed by Eckhardt's method.

The MICs of sodium arsenite and sodium arsenate for the transconjugants and transformants were (mM): 17 and 150 for *P. fluorescens* 38a (pBS3031), 17 and 400 for *P. putida* 53a (pBS3031), and 17 and 300 for *P. aureofaciens* BS1393 (pBS3031), respectively.

The original and plasmid-containing rhizospheric pseudomonads were used for inoculating seeds of sorghum, to test for bacterial effects on plants grown on soil contaminated with sodium arsenite.

The leaf tips of uninoculated control plants began to yellow and to wilt on day 7; the plants inoculated with the original pseudomonads, on day 14; and the plants inoculated with the plasmid-containing pseudomonads, on day 21. The plants that survived then developed necrotic spots on their leaves. At about 30 days after the start of the experiments, the plants stopped growing. The numbers of viable plants were estimated once a week. Uninoculated control plants began to die on day 14 (Fig.1). With 100 mg/kg of As, the last plants died on day 40; with 75 mg/kg of As, on day 60. When grown with 75 mg/kg of As, the plants inoculated with the rhizosphere pseudomonads survived better. The plants inoculated with *P. putida* 53a and its plasmid-containing derivative grew and developed normally (Fig. 2). A similar picture emerged when plants were treated with the other strains (not shown). The best results were obtained with *P. aureofaciens* BS1393. With 75 mg/kg of As, no differences were observed between the original rhizospheric strains and the plasmid-containing derivatives.

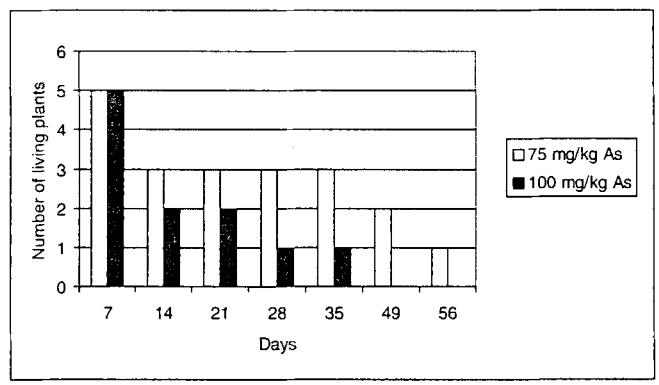

Fig. 1: Time course of the death of control (bacteria-untreated) plants

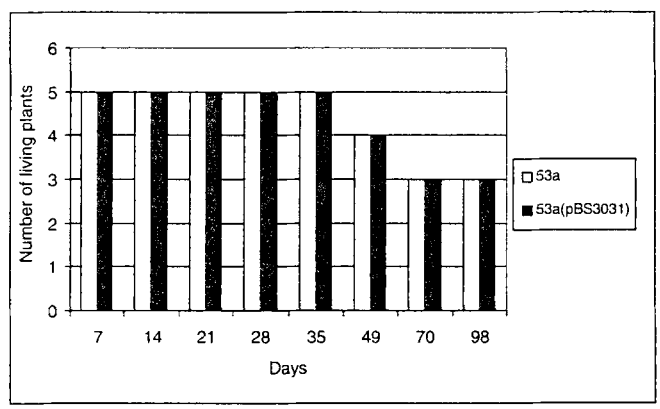

Fig. 2: Time course of the death of *P. putida*-treated plants grown on soil containing 75 mg/kg of As

However, there were differences with 100 mg/kg of As. On day 35, for example, the plants treated with the original *P. fluorescens* strain 38a survived better than did plants treated with its plasmid-containing derivative (Fig. 3). A similar picture was observed for plants treated with the other strains (Figs. 4 and 5).

In each experiment, we measured average plant-heights. With 75 mg/kg of As, the greatest heights were achieved by the plants treated with the plasmidless *P. putida* 53a; with 100 mg/kg of As, by the plants treated with the plasmidless *P. aureofaciens* BS1393 (Table 1). The plasmid-containing derivatives of these strains were not so stimulating to plant growth.

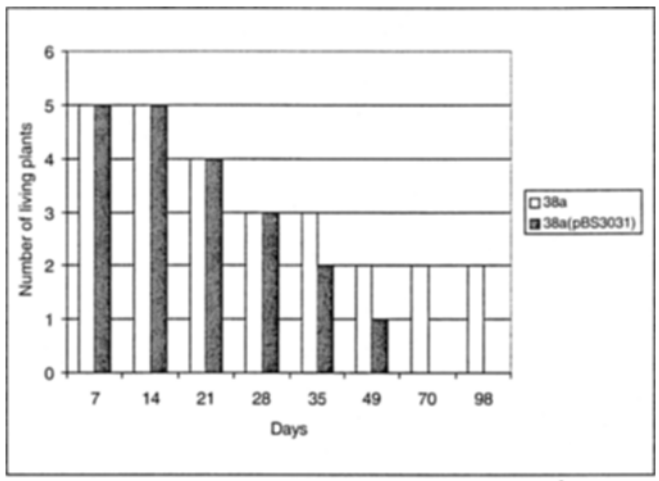

Fig. 3: Time course of the death of *P. fluorescens*-treated plants grown on soil containing 100 mg/kg of As

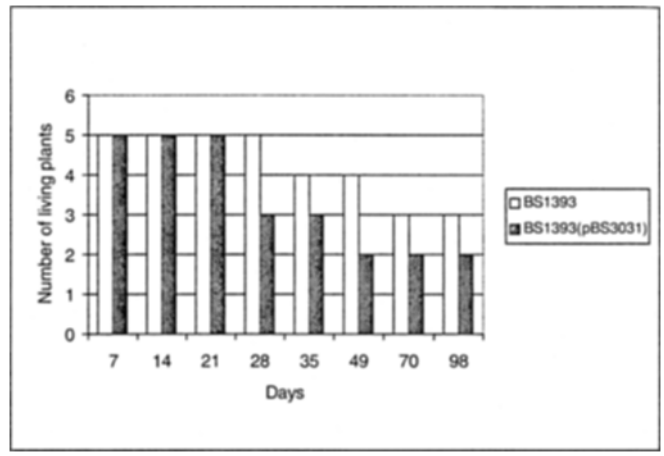

Fig. 4: Time course of the death of *P. aureofaciens*-treated plants grown on soil containing 100 mg/kg of As

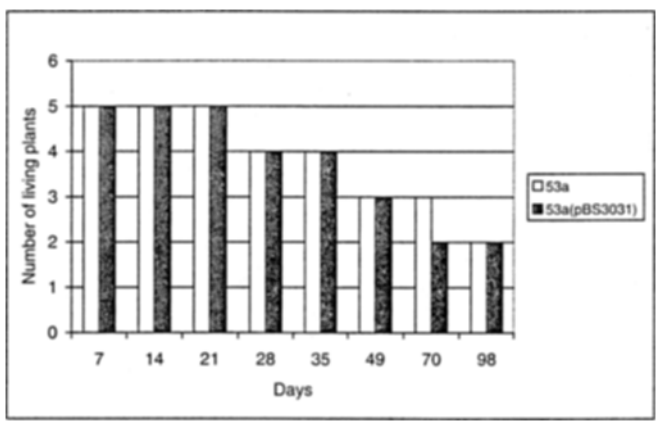

Fig. 5: Time course of the death of *P. putida*-treated plants grown on soil containing 100 mg/kg of As

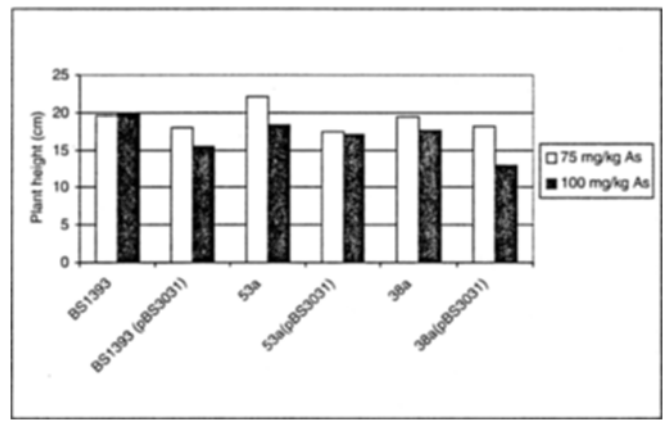

Fig. 6: Average heights (cm) of plants treated with the *Pseudomonas* strains used

Table 1: Average plant-heights (cm) measured after treatment with the *Pseudomonas* strains (day 98)

Strain	Height with 75 mg/kg of As	Standard deviation	Standard error	Height with 100 mg/kg of As	Standard deviation	Standard error
P. aureofaciens BS1393	19.6	2.3	1.02	20.0	2.3	1.15
P. aureofaciens BS1393 (pBS3031)	18.0	3.08	1.37	15.4	4.87	2.18
P. putida 53a	22.2	3.11	1.39	18.4	1.84	0.87
P. putida 53a (pBS3031)	17.4	2.5	1.12	17.0	4.96	2.48
P. fluorescens 38a	19.4	1.14	0.5	17.6	6.3	2.82
P. fluorescens 38a (pBS3031)	18.2	1.3	0.58	13.0	5.56	2.48

Table 2: Microbial-cell numbers per gram of root (estimated on day 98)

Strain	75 mg/kg of As	Standard deviation	Standard error	100 mg/kg of As	Standard deviation	Standard error
P. aureofaciens BS1393	$1.7*10^6$	2	0.89	$2.4*10^3$	3.8	1.7
P. aureofaciens BS1393(pBS3031)	$6.8*10^6$	2.3	1.02	$1.5*10^4$	5.65	2.52
P. fluorescens 38a	$4.3*10^5$	0.7	0.31	$2.5*10^3$	3.8	1.7
P. fluorescens 38a (pBS3031)	$7.7*10^5$	0.7	0.31	$1.4*10^4$	3.8	1.7
P. fluorescens 53a	$3.4*10^5$	0.7	0.31	$1.8*10^3$	3.08	1.37
P. fluorescens 53a (pBS3031)	$9*10^5$	1.4	0.63	$1.4*10^4$	3.8	1.7

The presence of plasmid pBS3031 in the rhizosphere strains slightly inhibited plant growth as compared with the effect produced by the plasmidless strains (Fig. 6). The slight inhibition was caused by all the plasmid-containing derivatives.

Analysis of the sorghum-rhizosphere populations of the *Pseudomonas* strains used in this study showed that the plasmid-containing derivatives were characterized by increased cell-numbers present on plant roots, in comparison with the original, plasmidless strains (Table 2). It is likely that plasmid pBS3031 ensures the selective advantage that the strains possessing this plasmid have in sodium-arsenite-containing soil.

3 Conclusions

- It is possible to increase the survivability of sorghum growing in sodium-arsenite-containing soil by using rhizosphere pseudomonads.
- The presence of the As(III)-/As(V)-resistance plasmid pBS3031 offers the strains a certain selective advantage in arsenite-contaminated soil.
- The presence of pBS3031 impairs plant growth due to the As-resistance mechanism determined by this plasmid: the transformation of the less toxic arsenate into the more toxic but water-soluble arsenite by arsenate reductase and the active removal of arsenite from bacterial cells.
- Such a mechanism makes it possible to develop a microbial-assisted phytoremediation technology for the cleanup of As-contaminated soils and is the only possible way of removing the soil-sorbed arsenates from the environment.

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