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ORIGINAL ARTICLE

Arsenic efflux in *Enterobacter cloacae RSN3* isolated from arsenic-rich soil

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Abstract

In the present study, bacterial isolates were screened for arsenic resistance efficiency. Environmental isolates were isolated from arsenic-rich soil samples (i.e., from Rajnandgaon district of Chhattisgarh state, India). Amplification and sequencing of 16S rRNA gene revealed that the isolates were of *Bacillus firmus RSN1*, *Brevibacterium senegalense RSN2*, *Enterobacter cloacae RSN3*, *Stenotrophomonas pavanii RSN6*, *Achromobacter mucicolens RSN7*, and *Ochrobactrum intermedium RSN10*. Arsenite efflux gene (*arsB*) was successfully amplified in *E. cloacae RSN3*. Atomic absorption spectroscopy (AAS) analysis showed an absorption of 32.22% arsenic by the RSN3 strain. Furthermore, results of scanning electron microscopy (SEM) for morphological variations revealed an initial increase in the cell size at 1 mM sodium arsenate; however, it was decreased at 10 mM concentration in comparison to control. This change of the cell size in different metal concentrations was due to the uptake and expulsion of the metal from the cell, which also confirmed the arsenite efflux system.

Introduction

Heavy metal contamination in the environment is a buzzing topic nowadays. Extensive growth in the industrial activities has led to the production of several heavy metal pollutants that contaminate soil and water, thereby harming our agroecosystem (Agrawal et al. 2015). Several novel physical and chemical strategies have been adopted for solving this problem, which ultimately proved to be costly and ineffective (Ayangbenro and Babalola 2017). Over few years, different new avenues of microbial bioremediation, i.e., bioaccumulation, biosorption, bioprecipitation, bioleaching, biotransformation, biosurfactants, and siderophore formation, have emerged as a new hope (Agrawal et al. 2018; Mosa et al. 2016; Kang et al. 2016). These eco-friendly methods are quite successful in addressing the issues lacking in their predecessors. It has reported that bioremediation can save up to 50–65% of the total cost as compared to conventional approaches (Ojuederie and Babalola 2017). Due to their widespread abundance, easy culturability, and varied mechanisms towards heavy metal tolerance, bacteria can be used as a suitable tool for heavy metal remediation.

A typical *ars* operon is generally transcribed as a single unit (Rosen 1999) containing either three (*arsRBC*) or five (*arsRDABC*) genes, which can be either plasmid or chromosome borne (Diorio et al. 1995). Sequential events and their associated genes involved in detoxification of arsenic are *arsC*, encoding As^{5+} reductase responsible for the reduction of As^{5+} to As^{3+} in the cytoplasm (Ji and Silver 1992); *arsB*, encoding a membrane protein that functions as an As^{3+} pump using the membrane potential force (Tisa and Rosen 1990); and *arsR*, as a regulator of the *arsRBC* operon by repressing the transcription of this operon in the absence of As^{3+} (Wu and Rosen 1993).

Thus, in this way, *arsB* gene indirectly provides resistance to arsenate by converting it into arsenite, which is further detoxified to form monomethylarsenite (*MMAs(III)*), dimethylarsenite (*DMAs(III)*), and trimethylarsine (*TMAs*) (Shen et al. 2013; Kruger et al. 2013). It has reported that bacteria contain arsenite oxidizing gene and arsenite transporter gene or any one of them (Sanyal et al. 2016). In the present study, six arsenic-resistant bacterial isolates were screened from different arsenic-contaminated sites of Chhattisgarh. A molecular approach was employed to decipher the arsenic resistance mechanism and the bioremediation potential of these native isolates.

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Materials and methods

Collection of soil sample and isolation of As-resistant bacteria

Surface soil samples (0–15 cm) were collected from Rajnandgaon district of Chhattisgarh, a state in central India. This state was earlier reported for heavy arsenic content in the soil. All samples (250 g) were collected in sterile plastic bags and refrigerated until further use. The soil samples were serially diluted up to 10^{-4} plated on nutrient agar (NA) plates containing 1 mM concentrations of sodium arsenite ($NaAsO_2$). The plates were incubated at 37 °C for 48 h to 72 h until colonies developed. Colonies with distinct morphology were further screened for sodium arsenate tolerance.

Determination of minimum inhibitory concentration

Flasks containing minimal broth (Dextrose, 1 g/L; dipotassium phosphate 7 g/L; monopotassiumphosphate, 2 g/L; sodium citrate, 0.5 g/L; magnesium sulphate, 0.1 g/L; ammonium sulphate, 1 g/L) were amended with different concentrations of heavy metal (HM) salt; sodium arsenate (20, 40, 50, 100, 200, 400, 600 and 700 mM). Single colony (CFU) from a pre-streaked plate of each isolate was inoculated with different concentrations of respective HM and incubated at 37 °C for 72 h. The growth of each isolate was determined spectrophotometrically by measuring the optical density (O.D.) at 600 nm.

Isolation of genomic DNA

Six bacterial isolates (RSN1, RSN2, RSN3, RSN6, RSN7, and RSN10) were revived in nutrient agar medium in the presence of 1-mM sodium arsenate (Na_3AsO_4) by the streak plate method. This concentration (1 mM) was previously by many researchers for initial screening of arsenic resistance bacteria (Banerjee et al. 2011; Selvi et al. 2014). Briefly, 100 μL of inoculum (0.6 OD overnight grown culture) was inoculated in nutrient broth (with 1 mM Na_3AsO_4) and incubated at 37 °C in a BOD incubator with constant shaking at 100 rpm for 12 h after that genomic DNA was isolated using Genomic DNA Isolation Kit (Himedia Laboratories Pvt. Ltd., Mumbai, India).

Amplification of 16S rRNA gene and in silico analysis

The isolated genomic DNA of all bacterial isolates were amplified for 16S rRNA gene using a set of universal primers U1 (5′-CCAGCAGCCGCGGTAATACG-3′) and U2 (5′ ATCGGCTACCTTGTTACGACTTC-3′) originally designed by Lu et al. (2000). The PCR mixture (25 μL) was composed of 1 μm of each primer, 2.5 mM of dNTPs, 1 U Taq polymerase, PCR buffer with 2.5 mM $MgCl_2$, and 1 μL of template DNA. The PCR reaction was carried out in a T100TM thermal cycler (Bio-Rad Laboratories, Inc.) with an initial denaturing temperature of 94 °C for 10 min followed by denaturing temperature of 94 °C for 1 min, annealing temperature of 55 °C for 1 min, extension temperature of 72 °C for 2 min and with having a final extension temperature of 72 °C for 10 min. The amplified products were partially sequenced by Sanger sequencing technique, and the gene sequences were deposited in NCBI GenBank. Furthermore, the BLAST algorithm (NCBI BLAST) was used to compare the gene sequences with the known ones to find their level of identity. The sequences were aligned using ClustalW, and their phylogenetic relationship was established by constructing a phylogram through neighbor-joining method in MEGA X.

Antibiotic susceptibility test and silver nitrate assay

All the cultures were studied for their co-tolerance towards heavy metals and antibiotics. The cultures were first grown in nutrient broth with 1 mM Na_3AsO_4 overnight for 16 h and after that 100 μL culture was evenly spread over nutrient agar plates having the same concentration metal along with HiPer Antibiotic Sensitivity discs (HiMedia Laboratories Pvt. Ltd., India) consisting of eight antibiotics. Silver nitrate assay was performed for arsenic-resistant bacterial strains to check the As(III) to As(V) and As(V) to As(III) transformation abilities of the isolates. The entire experiment was performed in duplicate (Simeonova et al. 2004; Selvi et al. 2014).

PCR amplification of *arsB* gene

The genomic DNA of all bacterial isolates were used for amplification of arsenite efflux gene *arsB* through a set of primers, i.e., darsB1F (5′-GGTGTGGAACATCGTCTGGAAYGCNAC3′) and darsB1R (5′-CAGGCCGTACACCACCAGRTACATNCC-3′), originally designed by Achour et al. (2007). The polymerase chain reaction protocol for *arsB* was performed in a 25 μL mixture composed of 1 μm of each primer, 2.5 mM of dNTPs, 1 U Taq polymerase, PCR buffer with 2.5 mM $MgCl_2$, and 1 μL of template for 35 cycles (denaturation for 45 s at 94 °C, annealing of 50 °C for 45 s, extension of 72 °C for 50 s, and final extension of 72 °C for 10 min after completion of an initial denaturation of 94 °C for 5 min).

Bioremediation assay

The selected isolate was grown in nutrient broth (Dey et al. 2016) in the presence (1 mM Na_3AsO_4) and absence of the arsenic salt along with a control (uninoculated). It was continuously shaken (at 100 rpm) for 7 days, and its growth was

periodically monitored in every 24 h at 600 nm in a Vis-spectrophotometer (Systronics India). Simultaneously, the environmental isolate was also grown in 100 ppm (0.5 mM) Na_3AsO_4 along with positive control (no metal), and samples were drawn on the 8th day (in decline phase) for AAS analysis. The sample was centrifuged at 6000 rpm for 5 min, and then the cell-free supernatant was processed for AAS analysis.

Scanning electron microscopy analysis

The best bacterial isolate was selected for cell size differentiation analysis using SEM. The culture was grown in minimal media with 1 mM and 10 mM metal salt concentrations; however, culture without metal salt was treated as control. The cells were fixed with 2.5% glutaraldehyde in 0.1-M phosphate buffer (pH 7.2) for 24 h at 4 °C and 2% aqueous osmium tetroxide post-fixed for 4 h. The processed samples were then examined under the scanning electron microscope (model: FEI Quanta 250) at 30 kV with 30,000X magnification for detection of any morphological changes in shape, size, density, and volume of cells (work done at Central Drug Research Institute, Lucknow, India). Cell size was measured by the manual method, i.e., "Old Way by Hand" method. The scale bar indicates that the actual distance on the specimen (for that bar) is 1 μm. The bar distance was the same for all three SEM micrographs. The length of scale bar was measured in mm,

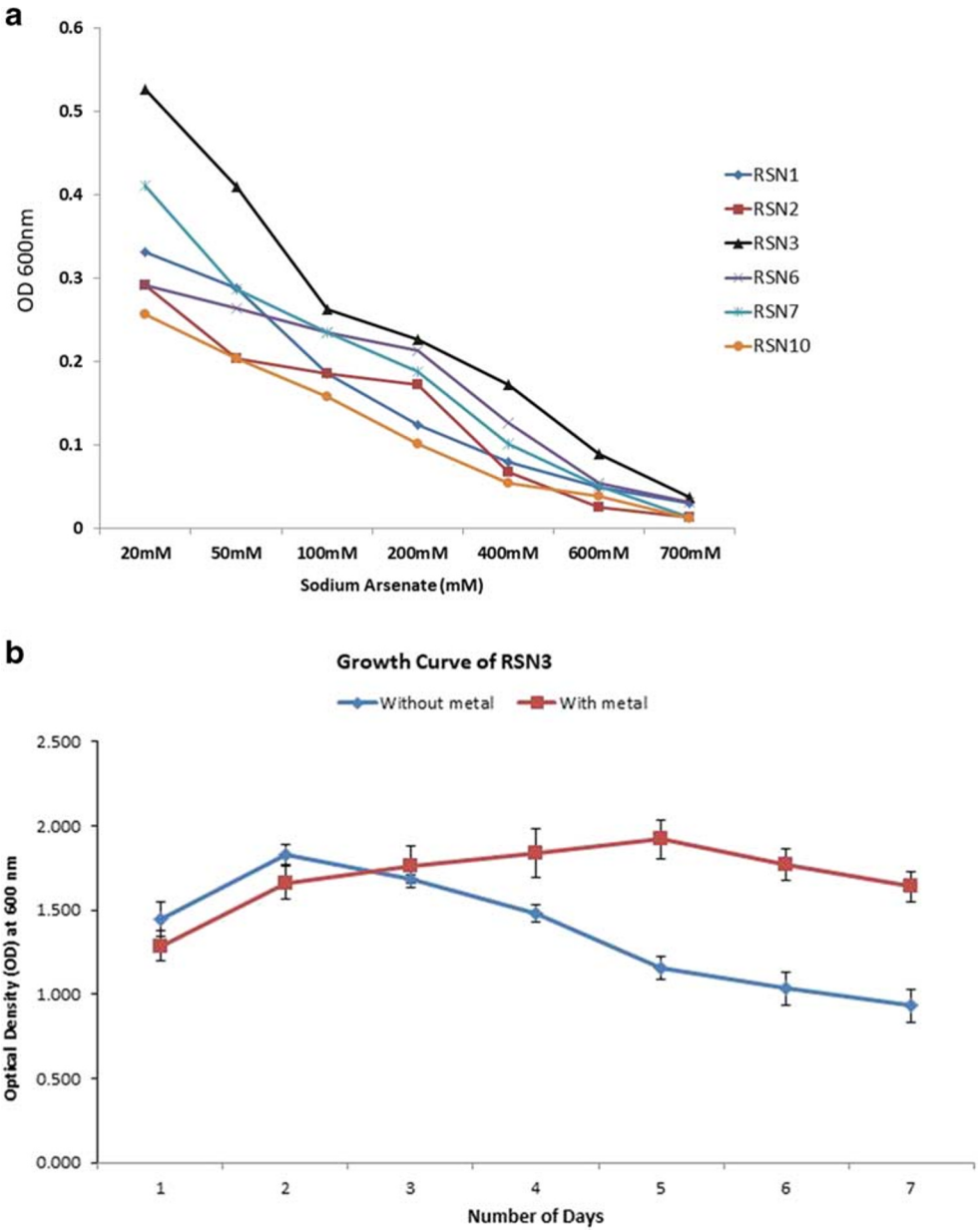

Fig. 1 **a** Minimum inhibitory concentrations of selected isolates grown in minimal broth with variable concentrations of metal salt. **b** Growth curve of RSN3 isolate in the presence and absence of arsenic (Na_3AsO_4). Each point represents the mean of three independent experiments. Bars, standard error of the means

which was considered to be equivalent to 1 μm. Similarly, cell sizes (5 cells / micrograph) were measured in mm and converted them into μm.

Results and discussion

Isolation of As-resistant bacteria

Only 20 bacterial isolates were obtained in nutrient agar medium containing 10 ppm arsenite. Most of the arsenic-resistant bacteria were obtained from soil samples from Rajnandgaon and, thus, suggested the presence of As in soil. Microbial resistance to arsenate [As (V)] and arsenite [As (III)] was determined by visible growth after 72 h in minimal broth supplemented with varying concentrations of sodium arsenate and sodium arsenite. Six bacterial isolates (RSN1, RSN2, RSN3, RSN6, RSN7, and RSN10) have shown growth on both the arsenic salts. The highest MIC was shown by isolate RSN3, i.e., 600 mM grown in minimal salt medium with sodium arsenate. This tolerance capacity is better than some previously reported environmental isolates (Anderson and Cook 2004; Wolfe-Simon et al. 2011; Bagade et al. 2016; Firrincieli et al. 2019). However, based on maximum tolerance, RSN3 was finally selected for further bioremediation studies (Fig. 1a).

Molecular characterization

The amplification of the 16S rRNA gene resulted in yielding approx. 996 bp DNA amplicons as reported previously by Lu et al. (2000). These sequenced amplicons were compared with the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for homology (Fig. 2)). All the identified genera's have already reported earlier showing resistance towards arsenic *Achromobacter* (Li et al. 2012; Bhosale et al. 2014), *Bacillus* (Dey et al. 2016), *Enterobacter* (Selvi et al. 2014), *Brevibacterium* (Sunitha et al. 2015), *Ochrobactrum* (Sarkar et al. 2014), and *Stenotrophomonas* (Bachate et al. 2009). The amplified products were partially sequenced, and the gene sequences were deposited in NCBI GenBank retrieving the following accession numbers, i.e., MG857853 (RSN1), MG857854 (RSN2), MG857855 (RSN3), MG857856 (RSN6), MG857857 (RSN7), and MG857858 (RSN10).

Antibiotics resistance and silver nitrate assay

The results obtained through antibiotic susceptibility test (Fig. 3) showed that all the isolates were resistant to the penicillin and rifamycin. Rifamycin (rifampin like an antibiotic) resistance occurred in various species of bacteria may be due to the modification in targeting this antibiotic. The *rpoB* gene codes for β-subunit of RNA polymerase (Padayachee and Klugman 1999). Among these six isolates, *Enterobacter cloacae* RSN3 and *Ochrobactrum intermedium* RSN10 had shown maximum tolerance for penicillin as it was the first generation antibiotic. However, *E. cloacae* was not earlier reported for these antibiotics resistance, but *O. intermedium* was previously reported as an extensive antibiotic-resistant against penicillin and rifampin (Johnning et al. 2013; Poszytek et al. 2018). Results exhibited tolerance developed by bacterial strain for older antibiotics. However, an increase in environmental pollutants may also be responsible for resistance towards antibiotics (Samanta et al. 2012). It has reported that heavy metals act as co-selecting agents in metal-resistant bacterial strains for the propagation of resistance in bacteria against antibiotics (Nguyen et al. 2019; Eduardo-Correia et al. 2020). Although the co-occurrence of heavy metal resistance and antibiotic resistance in the contaminated area was not clearly understood, the selection of either of the properties may characterize the genes for other.

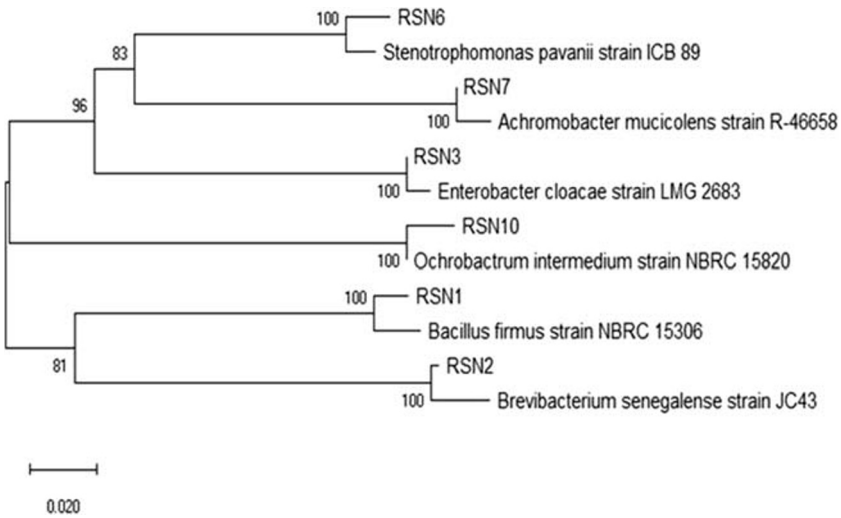

Fig. 2 Phylogenetic tree of arsenic-resistant bacterial isolates constructed by the neighbor-joining method using MEGA X. The scale bar indicates 2% nucleotide sequence substitution. The optimal tree with the sum of branch length = 0.73389547 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches

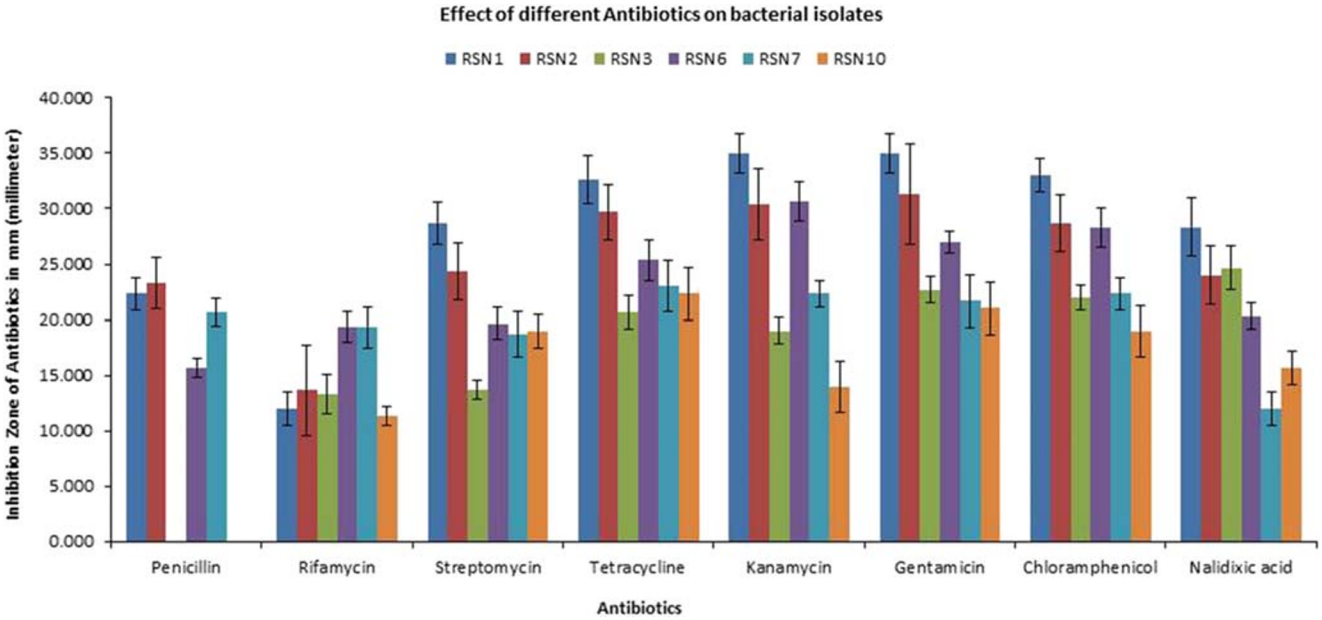

Fig. 3 Response of bacterial isolates to antibiotics represented by their inhibition zone (in mm). Each point represents the mean of three independent experiments. Bars, standard error of the means

Silver nitrate test resulted in the formation of a light yellow-colored precipitate, which revealed that all bacterial isolates were arsenate reducing bacteria. These results were in agreement with previous reports of Govarthanan et al. 2015 and Selvankumar et al. 2017.

PCR amplification of *arsB* gene

The *arsB* gene was successfully amplified from RSN3 bacterial isolate, i.e., *E. cloacae RSN3* out of six isolates. The amplicon was of approx. 750 bp in size as shown in Fig. 4, and it was the

Fig. 4 PCR amplified arsB gene (arsenite efflux gene) with approx. 750 bp

expected size according to Achour et al. 2007. The *arsB* is mainly code for an arsenite efflux gene that can either work independently or in association with *arsA* gene (Dey and Rosen 1995). We were able to amplify this gene from chromosomal DNA of the bacteria; however, this gene was found both in chromosome and plasmid (Butcher et al. 2000). Further, ArsB is an integral membrane protein able to evict arsenite from the cell cytoplasm, thus diminishing arsenite accumulation in the cell (Yang et al. 2012). The earlier experiments conducted in other bacteria explained that ArsB functions more efficiently when it works in association with ArsA (Rosen 2002). Interestingly, *arsBC* gene pair was common in the chromosomes of Gram-negative bacteria and chromosomes and plasmids of Gram-positive bacteria, but in the latter case, no *arsA* gene was present. As RSN3 is also a Gram-negative bacterium, and this information also confirmed the presence of *arsA* gene in RSN3 (Ben Fekih et al. 2018). These findings also revealed that *arsB* gene in *E. cloacae* works independently without its association with *arsA*. It means that the presence of *arsB* gene gives some insight about the probable mechanism of arsenic resistance in *E. cloacae RSN3*. On the other hand, arsenic resistance genes were characterized by several *Enterobacteriaceae* family members, but we found very few publications related to the amplification *arsB* gene from *E. cloacae* (Saltikov and Olson 2002).

Growth in the presence of As

Results exhibited that exponential phase of isolate RSN3 was very short (i.e., of only 24 h) when allowed to grow without heavy metal salt, but after 24 h it moved towards declining phase (Fig. 1b). In contrast, it has extended log phase of 3 days with a short stationary phase in the presence of arsenic (As) after that the declining phase starts from the 5th day onwards. The similar finding was also reported by Abbas et al. (2014) and reported that the stationary phase in non-stressed condition was longer than stressed condition (in presence of As). Results of AAS samples analysis revealed that RSN3 was able to absorb 32.22% arsenic (i.e., around 1/3 reduction of arsenic from control), which was quite good, and thus, it may be effectively used as a bioremediation tool in future.

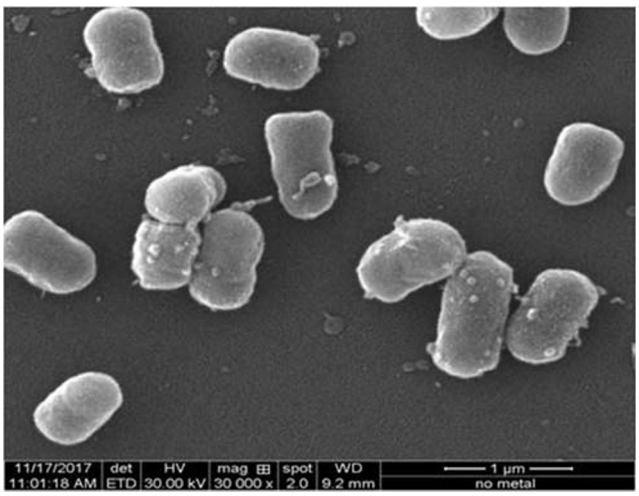

Fig. 5 SEM at × 30,000 with no arsenic

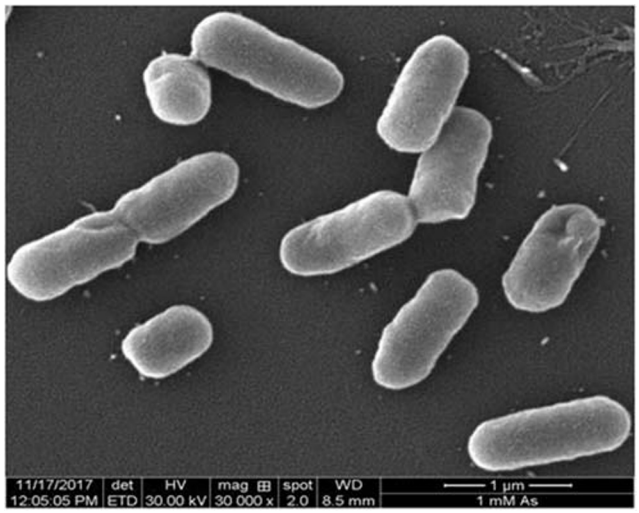

Fig. 6 SEM at × 30,000 with 1-mM Na_3AsO_4

Scanning electron microscopy

The bacterial cells recovered after growth curve experiment were observed at higher magnification (i.e., at × 30,000) for any morphological changes (Fig. 5, 6, and 7). Results showed a significant increase in the cell size (2.01 µm) as compared to control (1.24 µm) having no metal at 1-mM concentration. This suggested that there was an accumulation of arsenic inside the bacterial cell while growing in the presence of metal (Table 1). Interestingly, with a further rise in the metal concentration up to 10 mM, a noticeable decrease (1.51 µm) in

Fig. 7 SEM at × 30,000 with 10-mM Na_3AsO_4

Table 1 Cell length variations observed in SEM microgram

Treatment no.	Treatment details	Average length (μm)
T1	No metal	1.24
T2	1-mM sodium arsenate	2.01
T3	10-mM sodium arsenate	1.51
CD (1%)		0.169

the cell volume was observed. It was already confirmed from PCR amplification that the bacterium has *arsB* gene, which was responsible efflux of arsenic. ArsB mediated a primary arsenite pump in *E. coli* also (Yoon 2005). Similarly, morphological changes like an increase in cell size and shape were reported in *Acidocella* bacterium as a defense mechanism to reduce the heavy metal stress (Chakravarty et al. 2007). It has reported that some bacteria also form a chain like an arrangement in the presence of metal (Dey et al. 2016) and sometimes they decrease their surface area (Mohamed and Farag 2015). Further, our SEM finding also revealed that the change of cell length was due to the arsenic stress in *E. cloacae.*

Conclusion

The results obtained from PCR amplification and scanning electron microscopic studies indicated the presence of arsenite efflux system in *E. cloacae* RSN3 through which reduced arsenic extruded from the cell through *arsB* pump. Moreover, atomic absorption spectrophotometer analysis showed absorption of 32.22% of arsenic from the culture medium by this bacterium, which was quite significant, and therefore its arsenic resistance property can be further exploited for bioremediation purpose. Meanwhile, antibiotic susceptibility test revealed that this bacterium was resistant towards most of the antibiotics (particularly older generation antibiotics) besides showing resistance towards heavy metal (arsenic), therefore displaying co-resistance and cross-resistance towards these two components, i.e., antibiotics and heavy metals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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