Research Article

Isolation and identification of metal-tolerant and antibiotic-resistant bacteria from soil samples of Cachar district of Assam, India

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Abstract
The present study aims to assess the physicochemical properties and prevalence of microbial communities in soils samples collected from different locations of Cachar district, Assam, India. Bacterial communities in the soil were screened by morphological, biochemical and 16S rDNA sequence analysis and were identified as *Bacillus megaterium*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Chromobacterium pseudoviolaceum*. High concentrations of toxic metals negatively affect bacterial growth, and therefore, the minimum inhibitory concentration of isolated bacteria was determined against Cd, Pb, Fe and Cu by agar dilution technique. Co-resistance of antibiotic was also determined, which demonstrated that most of the metal-tolerant isolates were resistant to Methicillin and Penicillin. However, *P. aeruginosa* showed resistance to other antibiotics such as Cefdinir, Ampicillin, Kanamycin, Rifampicin and Vancomycin. The development and evolution of antibiotic resistance in soil bacteria occurs very likely naturally as a result of unethical and non-scientific disposal of toxic substances and industrial discharge, which also includes heavy-metal effluents and other clinical by-products. Therefore, pragmatic measures must be taken to limit the spread of antimicrobial resistance across the environment and to reduce the incidence of healthcare-associated infections.

Keywords Toxic metal · Minimum inhibitory concentration · Antibiotic · Soil micro-organism · Agar dilution technique

1 Introduction

Soil contamination is caused by the presence of xenobiotic chemicals or other alterations in the natural soil environment. Contamination is typically caused by the accumulation of heavy metals and metalloids through emissions from the rapidly expanding industrial areas, disposal of high metal wastes, leaded gasoline and paints, etc. Application of fertilizers, sewage sludge, pesticides, wastewater irrigation, coal combustion residues, spillage of petrochemicals and atmospheric deposition from smelting also poses a threat to environmental sustainability [1]. Many studies demonstrated that soil pollution directly affects human health. Risks to human health arise from the contamination of elements such as arsenic, lead and cadmium, organic chemicals such as PCBs (polychlorinated biphenyls) and PAHs (polycyclic aromatic hydrocarbons) and pharmaceuticals such as antibiotics [2].

Heavy-metal concentrations highly influence the microbial communities by inhibiting metabolic functions and altering enzymes specificity. Heavy metals damage the cell membranes, disrupt cellular functions and denature DNA and proteins [3, 4]. Heavy metals may also interfere with oxidative phosphorylation and osmotic balance [5]. However, micro-organisms also have their innate-tolerant ability to get adapted to the harsh environmental conditions [6]. Among the various adaptation mechanisms, the most commonly reported are metal sorption, mineralization, uptake and accumulation, extra-cellular precipitation, enzymatic oxidation or reduction to a less toxic form, and efflux of heavy metals from the cell [7]. Metal-removal efficiency varies among the micro-organisms, which

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may be due to the genetic makeup of micro-organisms or the composition of the reduction medium [8]. Some studies showed that metals can trigger antibiotic resistance in the environment since the atmosphere favours the co-selection of metal and antibiotic resistance [9, 10]. Environmental pollution by heavy metals not only triggers co-selection processes but also increases the level of tolerance to antibiotics due to co-regulation of resistance genes. Heavy-metal ions are known to co-regulate genes responsible for antibiotic resistance and decrease antibiotic susceptibility [11].

Therefore, the aim of the present study was to isolate and identify indigenous soil bacteria from contaminated sites and to determine microbial tolerance to heavy metal and co-resistance with antibiotics. The present study was conducted in the Cachar district of Assam, India, during the month of Jan–March, 2018. The average temperature was 11–23 °C, humidity 62–74%, with no rainfall during the span of the study. The weather condition during the study was notably clear and mildly sunny with heavy fog during the night, which persisted till early morning.

2 Materials and methods

2.1 Collection of soil samples

Soil samples were collected from four different locations of Cachar district of Assam, India. The sampling sites were public grounds and uncultivable lands, located nearby paper industry, brick factories, garage and food industry. The nearby areas were not protected by nature reserve or any park, and therefore, no specific permission was required during sampling. At each sampling site, two soil cores were collected from the top horizon (5–30 cm depth) that is considered to be biologically active with a strong hydrocarbon odour. The collected sub-samples were thoroughly mixed with a spade, and pooled into one composite sample per site. After sampling, the soil samples were transferred to plastic bags which were sealed and stored in a cold room (at 10 °C). Soil analyses were performed at Krishi Vigyan Kendra (KVK), Cachar, which includes determination of bulk density (g/mL), particle density (g/mL), porosity (%), moisture content (%), soil pH, electrical conductivity, macronutrient (C, N, P and K) and micronutrient (S, Fe, Zn, B and Na) contents. Isolation and identification of bacteria were performed within 18 h of sample collection.

2.2 Isolation and identification of bacteria

Isolation and quantitative computation of bacteria from soil samples were performed by serial dilution technique [12]. One hundred microlitres of samples was spread onto nutrient agar plates and incubated at 37 °C for 24 h. The total bacterial count was determined by counting the colonies in the microprocessor colony counter. Individual distinct colonies were sub-cultured and were identified by colony morphology, Gram's staining and biochemical tests (indole production, methyl red, Voges–Proskauer, citrate utilization, starch hydrolysis, etc.) [13]. Genomic DNA was extracted from isolated bacterial cultures [14], and PCR amplification of 16S rDNA gene was achieved by 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [15]. Sequencing of 16S rDNA was carried out at Xcelris Labs Limited, Gujarat, India, using ABI 3730×l 96 capillary system using Big Dye Terminator v3.1 kit. The consensus sequence of the 16S rDNA gene was generated from forward and reverse sequence data using aligner software. BLAST search was performed to find the closest homologous sequence, and based on the maximum identity score the first five sequences were selected. The nucleotide sequence thus obtained was analysed by Geneious R8 software package (Biomatters Ltd., Auckland, New Zealand). The 16S rRNA gene sequences were aligned using Clustal-W, and the phylogenetic tree was constructed using PhyML [16].

2.3 Assessment of bacterial tolerance to toxic metals

Bacterial tolerance to cadmium (Cd), lead (Pb), iron (Fe) and copper (Cu) was determined by agar dilution method [17], which were added in the form of cadmium chloride, lead acetate, ferrous sulphate and copper (II) sulphate pentahydrate, respectively [18, 19]. The initial concentration of these metal salts in nutrient plates was 50 µg/mL, and bacterial growth was observed by streaking on respective plates. Metal concentration was progressively increased by 10–15 µg/mL on a fresh agar plate, and the MIC was noted when the isolates failed to grow on respective plates. The experiment was conducted separately for Cd, Pb, Fe and Cu, taking five replicates at each concentration.

2.4 Antibiotics sensitivity and resistance pattern

Association of metal resistance with antibiotic resistance has been previously reported by many researchers [20], and therefore, the isolates were tested against 12 antibiotics using Kirby–Bauer disc diffusion technique [21]. Standard antibiotic discs were procured from 'HiMedia' which includes gentamicin (50 µg), amikacin (30 µg), cefalexin (30 µg), methicillin (30 µg), tetracycline (30 µg), ceftriaxone (30 µg), ampicillin (25 µg), chloramphenicol (10 µg), amoxicillin (10 µg), kanamycin (5 µg), ofloxacin (5 µg) and cefixime (5 µg). Antibiotic discs were placed on freshly

prepared lawns of each isolate on Mueller–Hinton plates and incubated at 37 °C for 24 h. The diameter of each inhibition zone was measured, and the strains were classified as resistant (*R*), intermediate (*I*) or susceptible (*S*) following the standard antibiotic disc chart.

3 Results and discussion

3.1 Physical properties and fertility status of soil

Analysis of soil samples revealed that the mean pH of the soil is 5.67 ± 0.12, which is slightly acidic and conducive to rice cultivation. Lower pH often increases the solubility of toxic metal ions, which are sometimes toxic to plants [22]. Essential elements such as zinc and boron were found to be deficient, whereas iron was found to be high, in the study area. The available sulphur varied from 3.7 to 8.3 ppm, and organic carbon content ranged from 0.32 to 0.53%. Both were found to be deficient in the study area. The amount of available nitrogen (N), phosphorous (P) and potassium (K) in soil samples is demonstrated in Table 1, which indicates the mean fertility index (NPK) of the study area as Low-Medium–Low. There was no definite trend in the distribution of essential elements in the soil, down the depth. The amount of sodium was found to be higher at the deeper horizons, probably due to illuviation and sodium saturation. The soil porosity decreased with increasing depth. Electrical conductivity ranged from 0.12 to 0.18 mS/cm.

3.2 Identification of metal-resistant bacteria

A total of 57 isolates were recovered from four different locations. The culturable portion of the bacterial community appeared to be affected by the increasing concentration of each metal salts. Quantification of species diversity and studying their tolerance ability has been used to monitor the environmental hazards. Many researchers claimed that culturable soil bacteria are the most active and play a vital role in plant growth promotion and balancing soil nutrients [23]. The isolates which showed significant tolerance towards heavy metals were taken into consideration for identification.

Isolates designated as SO1 (site 1) and SO2 (from site 2) were gram-positive having opaque white colonies and did not show any pigmentation. Isolates SO3 (site 3) and SO4 (site 4) were gram-negative and showed pigmentation on the surface of culture media. Microscopic observation showed that all isolates were rod shaped, whereas sample SO4 was Coccobacillus. All strains were motile and aerobic. Biochemical test results demonstrated negative results for indole test, methyl red test and urease test. Positive results were observed for citrate test, nitrate reduction test, catalase test and gelatin hydrolysis. Samples SO1 and SO2 were positive for Voges–Proskauer test and starch hydrolysis; however, SO3 and SO4 showed opposite results (Table 2).

3.3 Molecular identification of bacteria

16S rDNA sequence of the isolated bacteria was aligned using BLAST-N algorithm. Results of BLAST-N demonstrated that the query sequence has 97–99% identity and 100% query coverage with the 16S rDNA of the bacterium recorded in the GenBank. Phylogenetic tree inferred the degree of relatedness between 16S rDNA sequence of the isolates with other closely related 16S rDNA sequences retrieved from the database (Fig. 1). Based on these data, the isolates were identified as *Bacillus megaterium* strain GCC-SO1, *Bacillus cereus* strain GCC-SO2, *Pseudomonas aeruginosa* strain GCC-SO3 and *Chromobacterium pseudoviolaceum* strain GCC-SO4, having GenBank accession numbers: MH109306, MH109312, MH109307 and MH109305, respectively. *Bacillus* sp. and *Pseudomonas* sp. are the most commonly reported bacteria in soil, and possess much higher tolerance to heavy metals and other substances that are ever present in the environment [10, 24–28]. Dinucleotide repeats of eight bases (GCGCGCGC) were observed in all *Bacillus* sp. when compared with the aligned consensus sequence. On the other hand, seven nucleotide repeats (GTGGCGAGTGGCGA) were observed from position 89–104 bases of sample SO4 and few other *Chromobacterium* sp. A large deletion of nine bases (186–195 bases) was also observed *Pseudomonas* sp. and *Chromobacterium* sp. A higher GC proportion was observed in all the individual 16S rDNA sequences. The GC % of isolate SO1, SO2, SO3 and SO4 was 53.62%, 53.62%, 54.34% and 56.18%, respectively. These variations in GC contents often guide in finding phylogenetic relationships among genus and species [29].

3.4 Minimum inhibitory concentration

Environmental contamination by toxic substances exerts selective pressure on soil microbes and thus leads to the development of resistance systems to virtually all toxic metals [30, 31]. Metal-tolerant bacteria have evolved various resistance and detoxification mechanisms, and the ability to tolerate toxic metals is mostly plasmid-mediated [32]. Heavy-metal toxicity in the soil is dependent on the geographical location, bacterial diversity and metal concentration. The present study demonstrates that *Pseudomonas* sp. can withstand a high level of environmental contaminants and toxic substances such as heavy metals and antibiotics. Heavy-metal tolerance by *Pseudomonas* sp. and *Bacillus* sp. has been reported by many researchers.

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Table 1 Site description and soil fertility status

	Soil sample sites	GPS coordinates	Depth of sampling area (cm)	Bulk density (g/cm^3)	Particle density (g/cm^3)	Porosity test (%)	Soil moisture content (%)	Soil pH	Electrical conductivity (mS/cm)	Organic carbon content (%)	Available nitrogen (kg/ha)	Available phosphorous (kg/ha)	Available potassium (kg/ha)	Sulphur (mg/kg)	Iron (mg/kg)	Zinc (mg/kg)	Boron (mg/kg)	Sodium (ppm)
Site 1	Agricultural field nearby industrial discharge site	24°51′24″N 92°36′15″E	0–15	0.86	1.29	33.33	16.32	5.82	0.15	0.48	126.8	24.6	108.4	4.2	8.5	0.60	0.30	11
			15–30	1.08	1.52	28.95	19.29	5.50	0.13	0.33	128.5	21.7	84.2	5.9	6.3	0.68	0.21	6
Site 2	Uncultivable land nearby paper industry	24°51′48″N 92°36′36″E	0–15	0.93	1.22	23.77	10.93	5.33	0.12	0.39	143.3	17.4	147.0	4.4	15.3	0.43	0.23	20
			15–30	1.14	1.49	23.49	12.42	5.17	0.17	0.32	130.7	20.3	278.7	3.7	9.5	0.52	0.25	13
Site 3	Agricultural field nearby brick industry	24°36′03″N 94°35′11″E	0–15	1.21	1.60	24.37	17.45	5.98	0.16	0.41	167.0	12.0	67.0	6.7	11.2	0.54	0.17	18
			15–30	1.38	1.78	22.47	14.23	5.53	0.18	0.39	110.4	14.3	73.4	5.5	8.8	0.46	0.20	12
Site 4	Agricultural field nearby C.G food industry	24°45′18″N 92°54′39″E	0–15	0.93	1.20	22.50	21.22	6.10	0.13	0.53	263.2	27.1	102.3	8.1	8.3	0.57	0.21	14
			15–30	1.19	1.37	13.14	20.21	5.92	0.15	0.48	221.3	19.0	87.0	8.3	7.0	0.54	0.16	9

Table 2 Morphological and biochemical characteristics of isolated bacteria

	Sample SO1	Sample SO2	Sample SO3	Sample SO4
General characteristics				
Colony characteristics	Circular, opaque	Spread, irregular colony	Moist, translucent	Circular, raised
Size	Large	Large	Small	Small
Pigmentation	–	–	Yellow	Violet
Colour	White colonies	White colonies	Yellow–green colonies	Violet colonies
Margin	Entire	Wrinkled	Entire	entire
Motility	Motile	Motile	Motile	Motile
Growth condition	Aerobic	Aerobic	Aerobic	Aerobic
Gram staining	Gram-positive, rod shape	Gram-positive, rod shape	Gram-negative, rod shape	Gram-negative, Coccobacillus
Biochemical characteristics				
Indole	–	–	–	–
MR	–	–	–	–
VP	+	+	–	–
Citrate	+	+	+	+
Nitrate reduction	+	+	+	+
Starch hydrolysis	+	+	–	–
Urease test	–	–	–	–
Catalase test	+	+	+	+
Gelatin hydrolysis	+	+	+	+
Oxidase	–	+	+	+

'+' Indicates positive results, and '–' indicates negative results for respective tests

Some of their strains also play an important role in the remediation of organic and inorganic contaminants from the environment [33–35]. In the present study, *B. cereus* strain GCC-SO2 showed a much higher degree of tolerance towards lead and copper with MIC of 2050 ± 50 and 1716.66 ± 76.37 µg/mL, respectively. The strains of *Bacillus* sp., however, failed to withstand a high concentration of cadmium. Maximum tolerance towards cadmium and iron was shown by *P. aeruginosa* strain GCC-SO3, lead by *B. cereus* strain GCC-SO2 and *B. megaterium* strain GCC-SO1, and copper by *B. cereus* strain GCC-SO2 (Table 3). A promising result on the biosorption capability of cadmium and aluminium by *Bacillus* sp. has been reported by Arivalagan et al. [15] and Dhanarani et al. [36]. *Chromobacterium pseudoviolaceum* strain GCC-SO4 isolated in the present study showed significant tolerance to all tested metals, which is evidenced by other researchers [37, 38]. However, very less studies have been carried out on the abilities of *C. pseudoviolaceum* and their long-term application in bioremediation of metal-polluted soil. Oladipo et al. [39] reported that metal uptake rates and intra-cellular accumulation of heavy metals exceed the rate of extra-cellular loss. The differential quantities of metals accumulation by various isolates differ with the cell wall structures, as well as the synthesis of metalloproteins [40].

3.5 Antibiotics sensitivity and resistance pattern of isolated bacteria

Heavy-metal ions are known to co-regulate genes responsible for antibiotic resistance and decrease antibiotic susceptibility [41]. It has been observed that all the isolated strains were resistant to Methicillin and Penicillin. *P. aeruginosa* strain GCC-SO3, however, showed resistance to Cefdinir, Ampicillin, Kanamycin, Rifampicin and Vancomycin. *B. megaterium* strain GCC-SO1, *B. cereus* strain GCC-SO2 and *C. pseudoviolaceum* strain GCC-SO4 showed an intermediate zone of inhibition against Azithromycin, Ceftriaxone, Cefdinir, Rifampicin, Polymyxin and Co-trimoxazole (Table 4). Environmental pollution not only triggers heavy-metal co-selection processes but also increases the level of tolerance to some antibiotics due to co-regulation of resistance genes [42]. The combined expression of antibiotic and heavy-metal resistance by *B. cereus* and *P. aeruginosa* may not be a chance phenomenon but rather a result of selection by heavy metal present in an environment [26, 36, 43]. Therefore, pragmatic measures must be taken to limit the spread of antimicrobial resistance across the environment and to reduce the incidence of healthcare-associated infections.

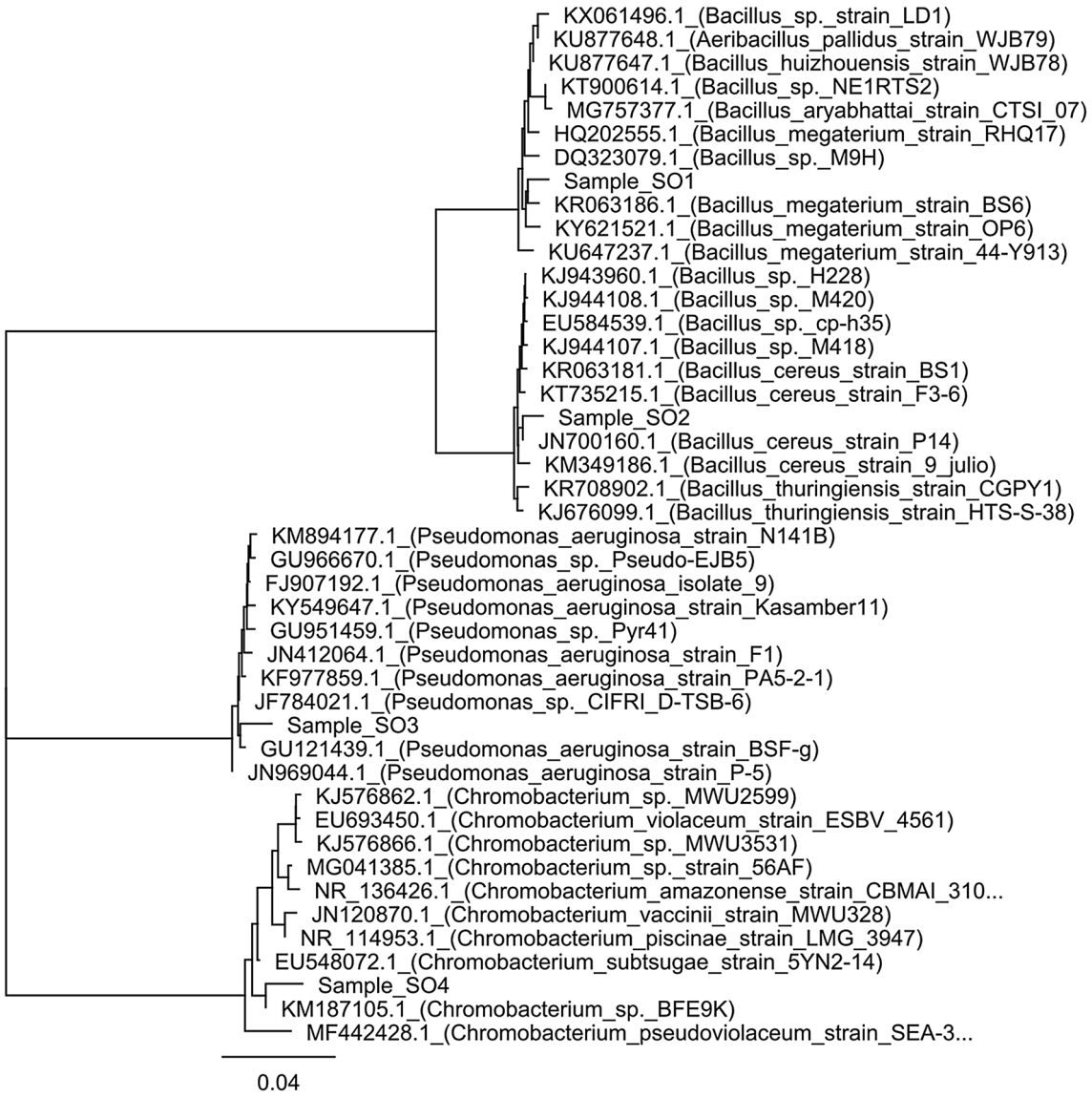

Fig. 1 Neighbour-joining tree, showing the phylogenetic relationship of sample SO1, SO2, SO3 and SO4 with closely related species

Table 3 Minimum inhibitory concentration of isolated bacteria against Cd, Pb, Fe and Cu (expressed in µg/mL of NA)

	Bacillus megaterium strain GCC-SO1	*Bacillus cereus* strain GCC-SO2	*Pseudomonas aeruginosa* strain GCC-SO3	*Chromobacterium pseudoviolaceum* strain GCC-SO4
Cadmium	33.33 ± 28.86	483.33 ± 76.37	1516.67 ± 76.37	1166.67 ± 57.73
Lead	1650.00 ± 50.00	2050.00 ± 50.00	966.66 ± 57.73	1350.00 ± 50.00
Iron	583.33 ± 28.86	716.66 ± 76.37	1633.33 ± 28.86	1183.33 ± 76.37
Copper	2383.33 ± 28.86	2533.33 ± 57.73	1716.66 ± 76.37	1683.33 ± 28.86

The values are the mean of three independent replications ± SE

Table 4 Antibiotic sensitivity and resistance pattern of isolated bacteria (expressed in mm)

Name of antibiotics	*Bacillus megaterium* strain GCC-SO1	*Bacillus cereus* strain GCC-SO2	*Pseudomonas aeruginosa* strain GCC-SO3	*Chromobacterium pseudoviolaceum* strain GCC-SO4
Methicillin	NI	NI	NI	NI
Azithromycin	18	15	9	14
Ceftriaxone	15	18	30	12
Cefdinir	14	11	NI	14
Co-trimoxazole	16	12	13	9
Ampicillin	10	9	NI	9
Ofloxacin	24	20	30	28
Ciprofloxacin	24	21	43	32
Norfloxacin	19	17	35	20
Kanamycin	18	7	NI	20
Amoxiclav	24	17	26	20
Meropenem	40	26	37	35
Streptomycin	25	20	22	22
Penicillin	NI	NI	NI	NI
Tetracyclin	28	18	14	27
Rifampicin	12	9	NI	13
Amikacin	23	20	19	23
Gentamicin	24	24	34	24
Polymyxin B	12	11	13	11
Vancomycin	15	12	NI	25

NI no inhibition zone; diameter of disc = 6 mm

4 Conclusion

The crop field nearby paper industry, brick factories, garage and food industry instigate microbial resistance towards toxic metals. The study demonstrates metal tolerance by *B. megaterium* strain GCC-SO1, *B. cereus* strain GCC-SO2, *P. aeruginosa* strain GCC-SO3 and *C. pseudoviolaceum* strain GCC-SO4. Isolated strains of *Bacillus* sp. exert maximum tolerance against lead and copper, and *Pseudomonas* sp. against cadmium and iron. *Chromobacterium* sp., however, showed a significant tolerance towards all tested metals. Co-selection of antibiotic resistance has also been observed, which may be the result of selection of heavy-metal tolerance by the isolated strains. Therefore, metal contamination represents a long-standing, widespread and recalcitrant selection pressure with both environmental and clinical importance that potentially contributes to the maintenance and spread of antibiotic resistance factors.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no competing interest.

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