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Metal Tolerance and Antibiotic Resistance of *Bacillus* species Isolated from Sunchon Bay Sediments, South Korea

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Abstract: Colony forming heterotrophic metal resistant bacteria was isolated from the sediments of Sunchon Bay, South Korea. All the isolates exhibit resistance to various heavy metals and also to wide spectrum of antibiotics. Based on the 16s rDNA sequencing the isolate were identified as *Bacillus* species and all the strains harbor a mega plasmid. Plasmid curing results in loss of antibiotic and heavy metals resistance in some of the isolates confirms a relationship between antibiotic and heavy metal resistance.

Key words: Sunchon Bay, heavy metals, *Bacillus*, plasmids, antibiotic resistance

INTRODUCTION

Heavy metals and their compounds are major pollutants in the coastal environment. The degree of contamination is associated with the natural geochemical features and anthropogenic inputs of the region. Presence of these heavy metals in the marine environment may pose a serious threat to the environment because of their ability to persist for several decades (Gochfeld, 2003).

Microorganisms are considered to be the best indicators of changes in environmental conditions (Bezverbnaya *et al*., 2005). In general, they are very sensitive to low concentration of heavy metals but rapidly adapt to the specific habitat conditions. An alternation of their activity may support the plasticity of communities and in some cases it ensures the possibility of melioration of the environment. However, it has been experimentally demonstrated that bacterial adaptation to heavy metal contamination in marine environment, can be accompanied by the spread of R-factors than by mutation and selection (Bhattacherjee *et al*., 1988). In plasmids, these heavy metal resistance genes may be linked with genes of antibiotic resistance and genes responsible for pathogenic properties (Bezverbnaya *et al*., 2005). Thus, individual bacterial strains have developed the capacity to survive under toxicological stress which is sometimes hazardous to human and animals. Hence, it is essential to study the metal resistant bacteria, which are being formed in response to selective anthropogenic pressure.

The Sunchon Bay is the well know brackish water ecosystem in South Korea and located in the southern part of the Korean Peninsula. The reed bed area of the bay links both the marine and inland environments, as well as a buffering zone to reduce the sudden changes from environmental stress. Such environmental features allow diverse life forms to thrive, resulting in high productivity and diverse biological functions such as natural decontamination. Recently, the Ministry of Environment, with the collaboration of the Ministry of Maritime Affairs and Fisheries and the authorities of Suncheon City, has been declared that the Suncheon Bay as a Wetland of International Importance. Currently, the domestic wastes and the water containing organic wastes were directly released into the reed bed system, which may contain heavy metals and other pollutants. This causes the microbes to be resistance to the heavy metals and antibiotics. Hence, the present study is aimed to isolate and characterize the metal resistant bacteria from Sunchon Bay sediments.

MATERIALS AND METHODS

Sediment sampling and bacterial isolation: Sediment samples were collected using Peterson grab (November 2006) and transported on ice to the Laboratory and processed within 10 h. Aerobic, cultivable bacteria were isolated by serially diluting 1 g of the sediment in sterile distilled water and 0.1 mL of the appropriate dilution were

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plated by spread plate technique on Luria Bertani (LB) Agar plates supplemented with 10 μg mL^{-1} of polymetals (Narita *et al*., 2003). Later, the plates were incubated at 26±2°C for 24 h and observed for bacterial growth. Morphologically distinct colonies were picked, purified and stored at 4°C for further analysis.

Evaluation of metal tolerance: Heavy metals like zinc, chromium, manganese, magnesium, mercury and cobalt were determined by agar dilution method (Kamala-Kannan *et al*., 2006). The lowest concentration of heavy metals at which no growth occurred when compared with the control plate was considered as the Minimal Inhibitory Concentration (MIC). All metal salts were added to the medium after autoclaving and cooling to 45 to 50°C from filter-sterilized stock solutions.

Identification of the metal resistant isolates: The metal resistant isolates were identified using 16s rDNA sequencing. The chromosomal DNA was extracted according to standard procedure of Maniatis *et al*. (1989) and the quality of the product was checked on 0.8% TBE Agarose gel.

The 16s rDNA was amplified using PCR with the universal primers 27f and 1492r (Reysenbach *et al*., 1992). The PCR products were cleaned using the QIAquick purification kit (Qiagen, USA). The amplicons were sequenced in both forward and reverse direction by using an automated sequencer ABI PRISM (Model 3700). The sequences were compared using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) for identification of the isolates. The nucleotides sequences were also deposited in the Genbank (Accession numbers: EF617315-EF617325).

Study of antibiotic resistance: Antibiotic resistance of the isolates was determined according to the Cruickshank (1968). Later, the plates were incubated at 26±2°C for 24 h and observed for bacterial growth. The lowest concentrations of antibiotics that inhibit the growth of the isolate compared with the control plate were defined as the MIC of the antibiotic.

Isolation and curing of plasmid DNA: *Bacillus* species were screened for the presence of plasmids according to standard procedure of Maniatis *et al*. (1989) with minor modification. Curing of the plasmids was performed according to Kamala-Kannan *et al*. (2006). To select the strains that lost antibiotic resistance, the colonies were transferred to LB agar plates amended with the respective antibiotics in appropriate concentrations (Ampicillin (Am) 20 μg mL^{-1}, Tetracycline (Tc) 5 μg mL^{-1}, Kanamycin (Kc) 5 μg mL^{-1} and Streptomycin (Sm) 5 μg mL^{-1}) under aseptic condition. The strains that had lost antibiotic resistance were then analyzed for the metal tolerance (Mercury 10 μg mL^{-1} and other metals 40 μg mL^{-1}).

RESULTS

Isolation and Identification of metal resistant bacteria: Eleven morphologically different heavy metal resistant bacteria have been isolated from the Sunchon Bay sediments. Polymerase chain reaction amplification of the targeted 16s rDNA resulted in the predicted 1.55 kb amplicons in all 11 isolates. The PCR amplified products were sequenced and compared with 16s rDNA sequences in NCBI database. Based on the partial sequences comparison by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) the isolates were identified as *Bacillus subtilis*, six strains designated as SB1, SB2, SB3, SB4, SB5 and SB6 *Paenibacillus favisporus*, two strains designated as SB7 and SB8 and the other species are *Bacillus cereus*, *Bacillus amyloliquefaciens* and *Bacillus licheniformis.*

Minimal Inhibitory Concentration (MIC) to heavy metals and antibiotics: Minimal inhibitory concentration of various heavy metals and antibiotics were determined for metal resistant bacteria isolated from Sunchon Bay sediments (Table 1). All the isolates exhibited high tolerance to manganese and less resistance to mercury. Among the isolates *B. cereus* exhibited high resistance to all the metals tested in present study. In antibiotic resistant assay all the isolates showed resistant to all the antibiotics tested (Table 1). In general, the isolates exhibit a maximum resistance to ampicillin, followed by tetracycline, kanamycin and streptomycin, respectively.

Table 1: Minimal Inhibitory Concentration (MIC) of various heavy metals and antibiotics (μg mL^{-1}) to the isolates

Bacteria	Hg	Co	Mg	Mn	Cr	Zn	Am	Tc	Sm	Kc
B. subtilis (SB1)	30	205	1300	1200	295	375	35	20	10	10
B. subtilis (SB2)	30	170	1300	1200	280	385	40	20	10	10
B. subtilis (SB3)	35	210	1300	1200	290	395	40	20	10	10
B. subtilis (SB4)	30	215	1300	1200	290	375	40	20	10	10
B. subtilis (SB5)	35	210	1300	1200	280	385	40	20	30	10
B. subtilis (SB6)	35	210	1300	1200	285	385	40	20	10	10
P. favisporus (SB7)	25	195	1300	1200	285	375	40	20	10	10
P. favisporus (SB8)	30	190	1300	1200	285	375	40	20	10	10
B. cereus	35	280	1300	1200	305	415	45	15	10	30
B. amyloliquefaciens	35	210	1300	1200	300	385	40	20	10	10
B. licheniformis	30	190	1300	1200	285	375	40	15	30	10

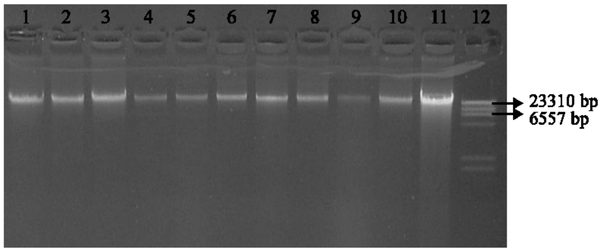

Fig. 1: Plasmid profile of the *Bacillus* species isolated from Sunchon Bay sediments subjected to electrophoresis on 0.8% Agarose gel. Lanes 1 through 11: *Bacillus subtilis* SB1, SB2, SB3, SB4, SB5 and SB6, *Paenibacillus favisporus*, SB7 and SB8, *Bacillus cereus*, *Bacillus amyloliquefaciens* and *Bacillus licheniformis* and Lane 12: *Hind* III digested lambda phage DNA as size marker

Plasmid profile and curing: Plasmid profile of the isolates exhibited a single band with the size of around 25 kb in all the isolates which indicates the presence of mega plasmid (Fig. 1). After curing, there was no loss in tetracycline resistance in all the isolates whereas the isolates became sensitive to kanamycin. In addition, some of the *B. subtilis* strains (SB1, SB2, SB3 and SB4) and *Bacillus amyloliquefaciens* were also became sensitive to streptomycin and ampicillin to which it was previously resistant. Whereas, for metals there was no loss in resistance to the aforesaid heavy metals in all the strains except for mercury, to which *B. subtilis* (SB3, SB6), *Paenibacillus favisporus* SB7 and *Bacillus licheniformis* strains showed sensitivity after curing, indicating plasmid-borne resistance.

DISCUSSION

Heavy metal resistant organisms have been isolated from the Sunchon Bay sediments. All the isolates were identified as *Bacillus* species and which is similar to the results of previous works reporting the presence of *Bacillus* species in metal contaminated environments (Kamala-Kannan *et al*., 2006, 2007). These strains were assayed for tolerance to various heavy metals on solid medium and resistance values may appear to be lower than typical reports obtained for the isolates from other aquatic ecosystem (Reyes *et al*., 1999; Kamala-Kannan *et al*., 2006, 2007). Several reasons could be accompanied for this firstly; the difference might be due to degrees of poly metallic pollution. Secondly, the type of organic constituents and presence of negatively charged ions like chloride in the medium may bind with the metal and alters the bioavailability and toxicity of metals resulting in differences in MIC of metal (Bezverbnaya *et al*., 2005).

The increased use of antibiotic and disinfectants in health care, heavy metals in industries creates the selective pressure for the survival of bacteria in a contaminated environment. Thus, in a multiple stressed environment, bacterial cells acquire these resistances by a change in the genetic makeup, either by mutation or by transfer of resistant genes between bacteria. Many investigators have also been reported the association between heavy metal and antibiotic resistance (Bhattacherjee *et al*., 1988; Lawrence, 2000; Verma *et al*., 2001). Similarly, in the present study also we found a relationship between heavy metals and antibiotic resistance.

Numerous studies have been reported that the bacterial flora present in the stressed environmental condition may carry plasmids not only in terms of the frequency but also in size (Hada and Sizemore, 1981; Baya *et al*., 1986). Tanaka *et al*. (1977) have reported the presence of large plasmids in *Bacillus* species, similarly in the current study a mega plasmid were observed in all isolates (Fig. 1). In order to check whether the heavy metals and antibiotic resistance was mediated by plasmids, plasmid curing was performed for all the isolates. Even after the curing, the isolates showed resistance to all the heavy metals tested, suggesting chromosomally encoded resistance whereas two strains of *B. subtilis* (SB3, SB6), *Paenibacillus favisporus* SB7 and *Bacillus licheniformis* showed susceptibility to mercury, indicating a plasmid-borne mercury resistance.

For antibiotics, some strains of the *B. subtilis* (SB1, SB2, SB3 and SB4) and *Bacillus amyloliquefaciens* became sensitive to streptomycin and ampicillin indicates the presence of resistance genes in mega plasmid. Interestingly, *B. subtilis* strain SB3 showed sensitivity towards ampicillin, streptomycin, kanamycin and mercury, which clearly confirm the association between the antibiotic and heavy metal resistance. Whereas, all the isolates exhibit resistant to tetracycline, illustrate the possibility of resistance gene in chromosomal DNA.

Thus the present work suggests that the brackish environment receiving a diverse anthropogenic input may provide a natural reservoir for the selection of poly resistant bacterial strains. All the strains were belonging to the genus *Bacillus* and they are the most dominant taxonomic groups in the communities of colony forming heterotrophic organism in heavy metal polluted environment. The resistance of the heterotrophic bacteria to metals and broad range of antibiotics, suggest that the some of the *Bacillus* species of Sunchon Bay may be hazardous to humans and marine animals as a consequence of natural selection of poly resistant forms and therefore, it needs further study.

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