J. Microbiol. Biotechnol. (1998), **8**(5), 497–500

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY

Incidence of Multiple Heavy Metal Resistance in a *Bacillus* Species

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Received: June 16, 1998

Abstract A new strain of *Bacillus* capable of growing upto pH 11 was isolated from a local pond polluted with detergents. This strain elicited unimpaired growth in media supplemented with heavy metals such as As, Cu, Cd, Hg, Ni and Zn. A MIC value of 200, 3, 1.60, 2.25, 7.50 and 3.70 mM was noticed for As, Cu, Cd, Hg, Ni and Zn respectively. Analysis of total DNA revealed the presence of a plasmid of 26 kb. This plasmid was lost by acriflavine treatment to the cultures. Such cured strains were found sensitive to heavy metals. Our findings suggest that incidence of heavy metal resistance is widely distributed and resistant strains could be isolated from heavy metal unpolluted sites.

Key words: *Bacillus*, heavy metal resistance, plasmids, survival

Heavy metal resistance is widespread among both gram-negative and positive bacteria [13, 20]. Bacteria respond to heavy metals in different ways depending on the species and the metal concentration in the environment. Toxicity is manifested by altered cell morphology, altered cell metabolism, bacteriostasis or lethality [6, 8, 18]. Silver and Mishra [15] reported that metal resistance in most bacteria studied intensively is more often a plasmid-mediated trait than chromosomally-coded function. Plasmid-mediated resistance to heavy metals is governed by a number of mechanisms depending on the type of metals and bacteria involved [11, 16]. Bacteria may survive in elevated concentration of toxic metals by employing detoxification mechanisms, including intracellular complexation, decreased accumulation and extracellular complexation [4, 5, 6, 13].

The proportion of resistant to sensitive microorganisms in different environmental samplings varies greatly but it is generally agreed that resistant cells occur frequently in the highly polluted sediments [1, 5, 20]. Though metal-resistant bacteria are not restricted only to metal polluted habitats, their frequency in these habitats tends to be quite high [2, 6]. Certain antibiotic-resistant bacteria also show resistance to metals [1, 2]. The question whether or not bacteria growing in unpolluted environments, or in habitats polluted with substances other than heavy metals, show resistance to heavy metals deserves some further study.

Many *Bacillus* spp. are known to grow in a variety of extreme environments and possess unique metabolic attributes [12]. However unlike other species of bacteria, comparatively less work has been done on metal resistance in *Bacillus* species [6, 12, 16]. In the present investigation we report metal resistance in *Bacillus* species isolated from a detergent-polluted pond.

MATERIALS AND METHODS

Organism and Growth Conditions

The test organism was collected from a pond (pH 8.5) near a detergent factory in Varanasi, India. The bacterium was isolated and purified employing standard microbiological techniques. Unless otherwise stated, cultures were grown in Yeast-Mannitol (YM) medium although it also showed appreciable growth in Luria-Bertani (LB) medium [17]. The taxonomic characteristics of the strain were determined following Bergey's Manual of Systematic Bacteriology [19].

Estimation of Growth

Growth was determined turbidometrically at 650 nm. Growth at higher pH was tested on a pH gradient plate. Suitable buffers Caps, carbonate-bicarbonate, were used to assess growth in liquid medium at various pH values.

Determination of Resistance to Heavy Metals

Toxicity and resistance to various heavy metals were tested by estimating percent survival on 1.5% agar (w/v) plates. Agar plates containing graded concentration of each heavy metal were prepared and thereafter equal

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inocula of the culture were plated. Plates were incubated in a bacteriological incubator at 37°C and the colonies appearing were counted after 72 h. MICs were recorded at the lowest metal concentration which prevented growth after 72 h. Every care was taken to use phosphate-free media while assessing growth, particularly with arsenate.

Uptake of Heavy Metals

The uptake of nickel and mercury by culture suspensions was determined as per the method of Slawson *et al.* [18]. Cells from an 18 h- grown culture were centrifuged and washed twice in 10 ml of 5 mM pipes buffer (pH 6.8), and harvested by centrifugation at 8,000×g for 15 min. Cell pellets were resuspended in 20 ml of pipes buffer and incubated at 37°C with shaking at 120 rev/min. Desired amounts (0.1 mM) of $NiCl_2$ or $HgCl_2$ were added to each flask, and 1 ml samples were periodically removed from duplicate flasks. Samples were centrifuged in an Eppendorf centrifuge at 15,000×g for 20 sec. The supernatant was removed and the cell pellet washed and resuspended in 1 ml of Pipes. The suspension was centrifuged again for 20 sec, the supernatant discarded, and to the resulting pellet, 1 ml 6 M ultrapure HNO_3 was added and the mixture held at 80°C for 2 h. Nickel and mercury uptakes were estimated by Atomic Absorption Spectrophotometery. Cell dry weights were determined by vacuum filtration as described earlier [10].

Plasmid Isolation

The method of Birnboim and Doly [3] was employed for plasmid isolation. Electrophoresis of plasmid DNA was performed on horizontal slab gel of 0.7% agarose. Ethidium bromide (2 μg/ml) was incorporated both in the gel and in the electrophoresis buffer. The gels were run at 150~200 V for 4~6 h.

Isolation of Cured Strains

Exponential phase-grown cultures were inoculated in a series of tubes containing two fold serial dilutions of neutral acriflavine in YM medium. The tubes were incubated for 48 h at 37°C. The culture treated with 12.5 μg/ml acriflavine showed only slight growth inhibition as compared to a control. It was diluted and plated on YM agar plates. After a 48 h incubation, isolated colonies were transferred to YM media and incubated for 72 h. Each culture was tested for resistance to heavy metals separately.

RESULTS

The organism was a polymorphic, gram-positive aerobic bacterium forming endospores. It was catalase positive, NO_3^- reducer, Voges-Proskauer positive and positive for starch hydrolysis. All these characters are reminiscent of a *Bacillus* sp. and accordingly we have tentatively identified the strain as a *Bacillus*.

Although the strain was isolated from a habitat of pH 8.5, it grew well both in acidic and alkaline media. Figure 1 shows its growth response at various pH values of the medium. Highest growth was recorded at pH 7 (4.5 h generation time), followed by pH 9, 6, 5 and 11. Prompted by the data of growth at a wide range of pH values, we became interested to check its growth in medium supplemented with NaOH (0.1 M). Initial pH of NaOH (0.1 M) containing medium before autoclaving was 12.63 but dropped to 8.90 after autoclaving. There was appreciable growth of *Bacillus* in the above medium after a few hour's lag phase. The pH of the NaOH-supplemented medium further dropped to 6.85 after growth of cultures for 72 h. Similarly the initial pH of 7 or 9 of control culture medium also dropped to 5.25 and 5.60 respectively after 72 h of growth.

In addition to its ability to grow at a wide range of pH, this strain grew in media supplemented with high concentrations of various heavy metals (Table 1), the highest level of resistance was to KH_2AsO_4 (150 mM). MIC value for different metals was exceedingly higher. Conceivably, this strain may have developed some resistance mechanism for growth in the presence of above metals. With a view to knowing more about this aspect, the uptake of Ni^{++} and Hg^{++} by the cells of

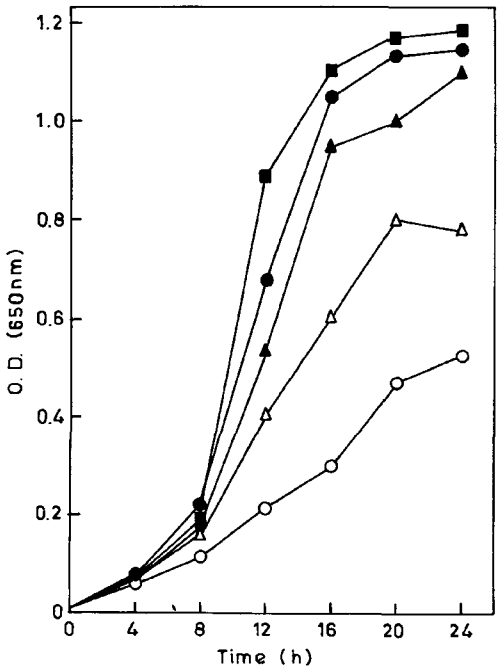

Fig. 1. Growth of *Bacillus* spp. at different pH of the medium: ■—■, pH 7; ●—●, 9; ▲—▲, 6; △—△, 5; ○—○, 11. Cultures were grown under identical conditions.

Table 1. MIC value of *Bacillus* spp. to heavy metals.

Metals	MIC (in mM)*	
	Parent strain	Plasmid-cured strain
KH_2AsO_4	200	50
$CuSO_4 \cdot 5H_2O$	3.0	1.0
$CdCl_2$	1.60	0.6
$HgCl_2$	2.25	0.8
$NiCl_2 \cdot 6H_2O$	7.50	1.0
$ZnCl_2$	3.70	0.8

*Concentration given is for metal salt.

Bacillus was estimated. Figure 2 shows that uptake of Hg^{++} and Ni^{++} by the cells of *Bacillus* did occur and the accumulation of Hg^{++} was comparatively higher than that of Ni^{++}. However, the cells accumulated only 2 to 3% of the added nickel or mercury (0.1 mM) from the medium. On the contrary heat-killed cells showed as low as 0.1% absorption of added Ni^{++} or Hg^{++} from the medium.

In many bacteria resistance to heavy metals is usually conferred by the presence of plasmids. DNA gel electrophoretic work on our isolate showed that a plasmid of approximately 26 kb was present in this organism (Fig. 3). Such a plasmid was routinely detected in cultures grown with or without heavy metals. Heat treatment of isolated plasmid did not alter the migration of band on gel, pointing to the covalently-closed-circular (CCC) nature of DNA.

To confirm whether the heavy metal resistance of the above strain was plasmid-coded, the cells were grown in the presence of acriflavine to eliminate the plasmids and then screened for heavy metal resistance. Sixty isolates were tested, and ten were found sensitive to heavy metals. All these transformed strains failed to grow in the presence of 1 mM or even lower concentrations of test metals excepting arsenate. It is evident from the data of Table 1 that the MIC value of cured strain to heavy metals

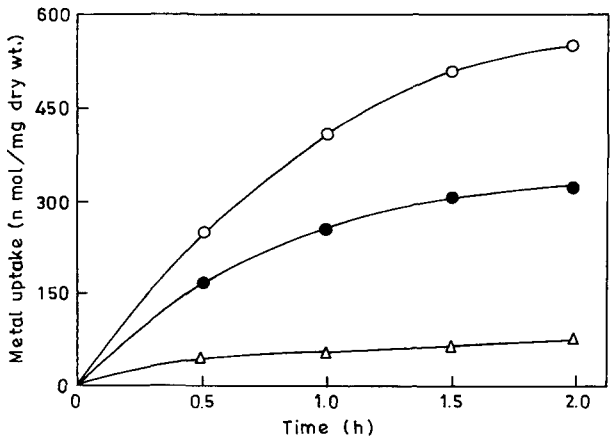

Fig. 2. Uptake of Hg^{++} (○—○) and Ni^{++} (●—●) by the cells of *Bacillus*. (△—△) uptake of Hg^{++} by heat-killed cells. Data are based on mean values from three identical experiments.

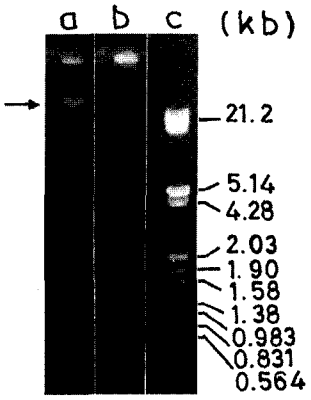

Fig. 3. Agarose gel electrophoresis of plasmid DNA isolated from *Bacillus* species: Lane a, plasmid DNA of culture grown without any heavy metal; b, plasmid DNA isolated from cultures grown with 150 mM KH_2AsO_4; c, standard λDNA digested with Hind III and EcoRI. Arrow on left side denotes the position of plasmid DNA band.

decreased drastically. Furthermore these strains did not show the presence of plasmid DNA band in agarose gel. In addition to above, plasmid DNA of metal resistant strain of *Bacillus* was found capable to transform metal sensitive strain of certain other *Bacillus* spp. to resistant one.

DISCUSSION

The morphological characters and biochemical attributes of the newly isolated strain attest to its resemblance with the well-characterised genus *Bacillus* [12, 19]. Several *Bacillus* sp. are known to grow at wide range of pH values and extensive biochemical work has been carried out on certain alkalophilic *Bacillus* spp. [7, 12, 17]. The strain employed in the present investigation also elicited growth at a wide range of pH values though the optimum growth occurred at pH 7.0. Since the strain was isolated from a pond having alkaline pH, most probable it has developed some adaptation mechanism to grow and thrive at higher pH side [9, 17].

Heavy metal resistant bacteria belonging to both gram-positive and negative groups have been isolated by several workers both from metal polluted and non-polluted habitats [2, 5, 20]. Duxbury and Bicknell [5] studied metal-tolerant bacterial populations from non-polluted and metal polluted soils in Australia and demonstrated that most bacteria in the metal-tolerant group were gram-negative and showed multiple antibiotic resistance. Our strain of *Bacillus* has been isolated from a pond not significantly polluted with heavy metals, and yet the strain showed resistance towards a number of heavy metals. Out of metals tested, this bacterium showed highest level of resistance to KH_2AsO_4 (150 mM). Bacteria resistant to high concentration of arsenate have been

isolated earlier [15, 20]. However our isolate showed resistance to quite high concentrations of a few other metals. Metal resistance in increasing frequency ranged from Cd<Zn<Hg<Cu<Ni<As. From our finding, it appears that this organism carries multiple resistance character for heavy metals.

Mechanisms governing heavy metal resistance have been studied in greater details in a number of bacteria [8, 11, 13, 15]. It has been clearly demonstrated that many bacterial strains contain genetic determinants of resistance to heavy metals. These resistance determinants are often borne on plasmids and transposons [2, 11, 13, 15]. Our work revealed the presence of a plasmid of approximately 26 kb in the metal resistant strain of *Bacillus*. The plasmid was not lost even when the cultures were grown in media free of heavy metals. However if these plasmid-bearing cells were treated with the curing agent acriflavine, the plasmid was lost. Such cured strains were found sensitive to heavy metals. Furthermore, the plasmid DNA of metal resistant strain showed efficient transformation ability in certain heavy metal sensitive strain of *Bacillus* spp. Transformation of other *Bacillus* species by pasmid DNA has been reported earlier [14]. We therefore conclude that resistance to heavy metal is governed by the presence of plasmid. Metal uptake experiments revealed that the strain is not permease defective, but accumulation of metal does occur at a reduced rate. This is in contrast to many other metal resistant bacteria which accumulate 50 to 70% of the added metals from the media [6, 8, 20]. The presence of plasmid together with the metal uptake potential of this strain suggest that it might have mechanisms such as the metal efflux system, and the metal reduction and volatization as reported in other bacterial strains. Further study may reveal the exact role of plasmid in determining the metal resistance. Our findings suggest that bacteria present in non-polluted (heavy-metals) habitat may also confer resistances to heavy metals.

Acknowledgments

Research work in the author's laboratory was supported by research grants received from Dept. of Biotechnology (No. BT/TF/02/03/90 & BT/R&D/02/15/94), Govt. of India, New Delhi. KLS is the receipient of Senior Research Fellowship of UGC, New Delhi.

REFERENCES

1. Andersen, S. R. and R. Sandaa. 1994. Distribution of tetracycline resistance determinants among gram-negative bacteria isolated from polluted and unpolluted marine sediments. *Appl. Environ. Microbiol.* **60:** 908–912.
2. Belliveau, B. H., M. E. Starodub, and J. T. Trevors. 1991. Occurrence of antibiotic and metal resistance and plasmids in *Bacillus* strains isolated from marine sediment. *Can. J. Microbiol.* **37:** 513–520.
3. Birnboim, H. C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acid Res.* **7:** 1513–1523.
4. Diaz-Ravina, M. and E. Baath. 1996. Development of metal tolerance in soil bacterial communities exposed to experimentally increased metal levels. *Appl. Env. Microbiol.* **62:** 2970–2977.
5. Duxbury, T. and B. Bicknell. 1983. Metal-tolerant bacterial populations from natural and metal-polluted soils. *Soil Biol. Biochem.* **15:** 243–250.
6. Gadd, G. M. 1992. Metals and microorganisms: A problem of definnition. *FEMS Microbiol. Lett.* **100:** 197–204.
7. Grant, W. D., W. E. Mwatha, and B. E. Jones. 1990. Alkaliphiles: Ecology, diversity and applications. *FEMS Microbiol. Rev.* **75:** 225–270.
8. Ji, G. and S. Silver. 1995. Bacterial resistance mechanisms for heavy metals of environmental concern. *J. Indust. Microbiol.* **14:** 61–75.
9. Kristjansson, J. K. and G. O. Hreggvidsson. 1995. Ecology and habitats of extremophiles. *World J. Microbiol. Biotech.* **11:** 17–25.
10. Kumar, A., F. R. Tabita, and C. Van Baalen. 1982. Isolation and characterization of heterocysts from *Anabaena* sp. strain CA. *Arch. Microbiol.* **133:** 103–109.
11. Mishra, T. K. 1992. Heavy metal bacterial resistances. *Encyclop. Microbiol.* **2:** 361–369.
12. Moo, B. and M. Y. Kim. 1997. A new alkalophilic bacterium producing ethylene. *J. Microbiol. Biotech.* **7:** 212–214.
13. Nies, D. H. 1992. Resistance to cadmium, cobalt, zinc and nickel in microbes. *Plasmid* **26:** 17–28.
14. Pragai, Z., A. Holczinge, and T. Sik. 1994. Transformation of *Bacillus licheniformis* protoplasts by plasmid DNA. *World J. Microbiol. Biotech.* **140:** 305–310.
15. Silver, S. and T. K. Mishra. 1988. Plasmid-mediated heavy metal resistances. *Ann. Rev. Microbiol.* **42:** 717–743.
16. Silver, S. and M. Walderhaug. 1995. Bacterial-plasmid-mediated resistances to mercury, cadmium and copper. pp. 435–458. *In* R. A. Goyer, and M. G. Cherian (eds.), *Toxicology of metals: Biochemical aspects.* Springer-Verlag, Berlin.
17. Singh, K. L. 1997. Physiological and biochemical studies on microbes isolated from certain harsh conditions. Ph.D. thesis, Banaras Hindu University, Varanasi, India.
18. Slawson, R. M., M. I. Van Dyke, H. Lee, and J. T. Trevors. 1992. Germanium and silver resistance, accumulation and toxicity in microorganisms. *Plasmid* **27:** 72–79.
19. Sneath, P. H. A. 1986. Endospore-forming Gram-positive rods and cocci. pp. 1104–1139. *In* P. H. A. Sneath, N. S. Mair and E. Shape (eds.), *Bergey's Manual of Systematic Bacteriology*, vol. II, Williams and Wilkins, Baltimore.
20. Trevors, J. T., K. M. Oddie, and B. H. Belliveau. 1985. Metal resistance in bacteria. *FEMS Microbiol. Rev.* **32:** 39–54.