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Biosequestration, Transformation, and Volatilization of Mercury by *Lysinibacillus fusiformis* Isolated from Industrial Effluent

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In the present study, an efficient mercury-tolerant bacterial strain (RS-5) was isolated from heavy-metal-contaminated industrial effluent. Under shake flask conditions, 97% of the supplemented mercuric chloride was sequestered by the biomass of RS-5 grown in a tryptone soy broth. The sequestered mercuric ions were transformed inside the bacterial cells, as an XRD analysis of the biomass confirmed the formation of mercurous chloride, which is only feasible following the reaction of the elemental mercury and the residual mercuric chloride present within the cells. Besides the sequestration and intracellular transformation, a significant fraction of the mercury (63%) was also volatilized. The 16S rRNA gene sequence of RS-5 revealed its phylogenetic relationship with the family Bacillaceae, and a 98% homology with *Lysinibacillus fusiformis*, a Gram-positive bacterium with swollen sporangia. This is the first observation of the sequestration and volatilization of mercuric ions by *Lysinibacillus* sp.

Keywords: Metal sequestration, biosorption, biotransformation, mercury, volatilization

Heavy metals are ubiquitous and persistent pollutants that are introduced to the environment through natural and anthropogenic activities, such as mining and smelting, as well as other sources of industrial waste [14]. Attracting more widespread attention as environmental pollutants, heavy metals enter the food chain through contaminated ground water and then move to higher trophic levels in the ecosystem [12]. Methods for removing heavy metals from contaminated sites include precipitation, filtration, ion-exchange, oxidation reduction, electrochemical recovery, membrane separation, and other techniques. However, the use of organisms to remediate contaminated sites has become a fast-growing and promising technology with several advantages over traditional methods [7].

Mercury (Hg) is a widely distributed toxic metal [19] and a serious pervasive environmental pollutant, as it accumulates in the tissues of animals and humans *via* the food chain [6]. Mercury vapor enters the bloodstream after being absorbed through inhalation and is then transformed to divalent mercury. Inhalation of mercury vapor at high levels can result in acute, corrosive bronchitis and interstitial pneumonitis. Mercury also affects the memory, and long-term exposure can lead to severe depression [14]. Mercury exists in different oxidation states and becomes part of the natural environment after its release either through adsorption to sediment particles in soil or in complexes with soil organic matter [16]. Metallic mercury [Hg(0)] is volatile, has a poor water solubility, and can be transported through the atmosphere [2]. Microorganisms interconvert Hg(II) and Hg(0), and CH_3Hg and Hg(0). Bacterial mercury resistance involves the biotransformation of toxic forms of mercury into a nontoxic form by conversion to a non-available form. The *Mer* operon confers the information and mechanism harbored by metal-tolerant bacteria towards the methylation of mercury. Numerous studies on the methylation of mercury in different ecosystems have already been reported in recent years, where all the studies were carried under anaerobic conditions. In fact, microbial Hg methylation is the only experiment that is routinely carried out under anaerobic conditions, as this process is known to take place in anoxic environments and under anaerobic conditions [22].

However, other transformations (*e.g.*, reduction, demethylation, and oxidation) can be carried out under aerobic or anaerobic conditions. The mercuric cation and

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oxyanion resistance mechanisms exhibited by certain bacteria are mainly based on efflux pumps or extracellular sequestration [15]. Accordingly, the present study examined the sequestration and transformation of Hg by aerobically growing bacterial cells. Two aerobic strains with potential to remediate mercury-contaminated sites were isolated. However, one strain was lost during the experimental trials owing to sudden exposure to a high temperature. The other strain, RS-5, that was named *Lysinibacillus fusiformis* based on its 16S rRNA gene homology, exhibited the ability to survive at high temperatures and accumulate mercury efficiently in natural environments. This is the first report on a *Lysinibacillus* sp. showing efficient mercury sequestration and biotransformation.

Materials and Methods

Enrichment and Isolation of Microorganisms

Industrial effluent was collected from metal-contaminated sites in Punjab and suspended in 100 ml of a tryptone soy broth (TSB) with the following composition (g/l): Peptone from casein 17.0 g, Peptone from soymeal 3.0 g, D (+) Glucose 2.5 g, NaCl 5.0 g, and K_2HPO_4 2.5 g supplemented with mercuric chloride (5 mg/l mercury equivalent), in 250 ml Erlenmeyer flasks. The flasks were incubated at 37°C on an incubator shaker (120 rpm) until growth appeared. A small aliquot (1 ml) was then transferred to a fresh medium containing mercury to enrich the mercury-tolerant bacteria. Thereafter, a loopful of each culture was streaked on tryptone soy agar (TSA) plates supplemented with mercuric chloride (5 mg/l) for purification. The plates were incubated under aerobic conditions at 37°C for 24 h. Isolated colonies with different morphologies were then picked up and streaked on fresh TSA plates at least three times to ensure an axenic culture. The isolates were then preserved on the TSA plates at 4°C until further study.

Multiple Metal Tolerance Studies

The bacterial isolates were further screened for their tolerance and resistance towards different metals. The axenic cultures were inoculated into TSB supplemented with different concentrations (*i.e.*, 5, 10, 25, and 50 mg/l as the active ingredient) of mercury (Hg), lead (Pb), cadmium (Cd), nickel (Ni), arsenic (As), tin (Sn), selenium (Se), zinc (Zn), chromium (Cr), and copper (Cu).

Growth Profiling

The selected isolate (RS-5) was exposed to 25 mg/l mercury as $HgCl_2$ in the culture medium to examine the growth profile under mercury-stressed conditions. The cells were inoculated into a tryptone soya broth supplemented with mercuric chloride and incubated on an orbital shaker at 120 rpm and 37°C, along with a control. The growth was monitored by measuring the optical density at 600 nm, every 24 h up to 96 h.

Biotransformation Studies

To check the biotransformation potential of RS-5 in terms of reduced and volatilized mercury after sequestration, the isolate was inoculated in TSB (Himedia) supplemented with 25 mg/l mercuric chloride. The organism was grown in round-bottom flasks including an inlet to supply fresh air and an outlet to facilitate collection of the spent air. A steady flow of fresh sterile air was maintained using bubbler pumps through presterilized 0.22 m membrane filters (Whatman, USA). Meanwhile, the spent air containing volatilized mercury components was trapped in acidic potassium permanganate (Merck, USA), as described elsewhere [9]. The trapping solution was replaced with a fresh solution every 24 h. Abiotic controls were also maintained under similar conditions in TSB supplemented with mercuric chloride. Furthermore, the Hg uptake by the isolate was determined after separating the cell pellet from the spent cell-free supernatant (CFS) every 24 h until the end of the experiment (96 h) to determine the mercury in the different fractions. The cell pellet and CFS were acid digested using premixed concentrated nitric acid (HNO_3) and perchloric acid ($HClO_4$) in a 3:1 ratio, followed by subsequent dilution, as described elsewhere [20], whereas the trapping solution was digested without further treatment. Thereafter, the samples were diluted with 0.2% HNO_3 and subjected to an ICP-MS analysis to estimate the mercury in the different fractions.

ICP-MS Analysis

The ICP-MS analysis to estimate the mercury in the different fractions was carried out using a Perkin Elmer SciexElan DRC-e (Axial Field Technology). For mercury, the system was operated under inert conditions maintained with argon gas at a flow rate of 15 L through plasma, plus 1.2 L and 0.9 L per minute from an auxiliary nebulizer throughout the analysis. The pressure was maintained between 60 and 70 psi. The plasma formation was carried out at 6,000°C, which caused the samples to separate into individual atoms (atomization), followed by ionization with the plasma and detection using a mass spectrometer.

Characterization of Intracellular Mercury

The crystallographic characterization of the bioaccumulated mercury was carried out using a Panalytical X'Pert Pro Powder X-ray diffractometer. The bacterial biomass grown in the TSB supplemented with 25 mg/l mercuric chloride was collected after centrifugation and then air dried in a conventional oven at 70°C until there was no further appreciable change in the weight of the biomass. The samples were then powdered using a pestle and mortar. The X-ray diffraction patterns of the powder samples were recorded using Cu K_α radiation ($\lambda = 1.541$Å) keeping a step size of $0.0017^o s^{-1}$ in a 2θ range of $20^o - 80^o$ at a generator tension of 45 kV and generator current of 40 mA. The resulting diffractograms were compared with a reference database obtained from COD-Iorg REV 22182 on the basis of the 2θ values, along with specific diffraction lines using Match software designed for powdered X-ray diffraction analysis.

Biochemical and Molecular Characterization of Isolate

Based on the transformation and volatilization potential of strain RS-5, its morphological and biochemical characteristics were explored. The biochemical characterization of this strain, henceforth referred to as *Lysinibacillus fusiformis*, was carried out at the Microbial Type Culture Collection (MTCC-IMTECH), Chandigarh, India. For the molecular characterization and phylogenetic relatedness of RS-5 to other organisms, its genomic DNA was isolated and partially amplified using standard protocols [17], followed by sequencing of the 16S rRNA gene. The PCR amplification was performed using universal primers (27F and 1492R), where the

initial denaturation was carried out at 95°C for 1 min for 1 cycle, followed by 37 cycles (denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min) and a final extension at 72°C for 15 min. The amplification of a 1.5 kb fraction was confirmed by agarose gel electrophoresis. The PCR product was then eluted, purified, and sent for sequencing at Chromous Biotech, Bangalore, India. The multiple sequence alignment was carried out using BLAST [1], followed by classification using RDP II Classifier tools and a phylogenetic analysis using MEGA 4 [8].

Statistical Analysis

The growth and transformation experiments were carried out in triplicate, and the results are presented as the mean values along with the standard error in the respective figures.

RESULTS

The present study examined the sequestration and transformation of Hg by aerobically growing bacterial cells. Two aerobic strains with the potential to tolerate mercury were isolated. One strain was subsequently lost during the experimental trials owing to sudden exposure to a high temperature. The other strain, RS-5, was found to be resistant to various metals up to 50 mg/l, and Cd and As up to 25 mg/l. Therefore, this isolate was further explored as regards the sequestration and volatilization of mercury.

Growth in Presence of Hg

The growth kinetics of the selected mercury-tolerant strain were monitored in terms of the optical density changes over a period of 96 h in a mercury-supplemented broth compared with a control that had no added mercury (Fig. 1). A small amount of growth was observed in the presence of Hg^{2+} ions during the first 24 h; however, a substantial exponential growth phase was noted in the mercury-stressed biomass after 24 h, indicating acclimatization of the cells. Between 24 h and 48 h of incubation, comparable growth was observed in the normal and mercury-supplemented broth. After 96 h of incubation, a higher optical density was measured in mercury-induced medium, possibly due to the slight silvery black coloration of the biomass.

Hg Sequestration and Volatilization

As regards the mercury sequestration and biotransformation (*i.e.*, reduction and volatilization) by *L. fusiformis*, the mercury in the biomass, cell-free supernatant, and trapping solution was quantified. When subjected to 25 mg/l of mercuric chloride, more than 97% of the mercury was transformed/volatilized by the isolate after 96 h, compared with 35% during the first 24 h. Between 48 and 96 h, simultaneous sequestration and volatilization of the mercury was observed with an almost fixed amount of mercury in the biomass. This observation also coincided with the appearance of a silvery black color in the centrifuged biomass, which likely resulted from the formation of elemental mercury loosely bound to the cell membranes. However, the volatilized fraction of mercury was significantly higher than the sequestered and reduced mercury inside the biomass, as more than 63% of the mercury was transformed into volatile mercury in the trapping solution during the time course of 96 h (Fig. 2).

Crystallographic Characterization of Sequestered Hg

In the recorded XRD pattern (Fig. 3), six major peaks were identified and assigned as 1 to 6 in the diffractrogram at the 2λ values: 21.42°, 28.16°, 32.83°, 43.80°, 46.29°, and 52.94°, respectively. All these major peaks were identical with the reference database available for calomel (File No. 96-9008978) in the literature [4]. A significant matching of other parameters associated with the X-ray diffractogram, like the d-spacing (Å) and relative intensity (%), was also observed between the reference data sheet and the present study (Table 2). Thus, the reference data confirmed the reduction of divalent mercuric ions into mercurous chloride with a tetragonal crystal lattice.

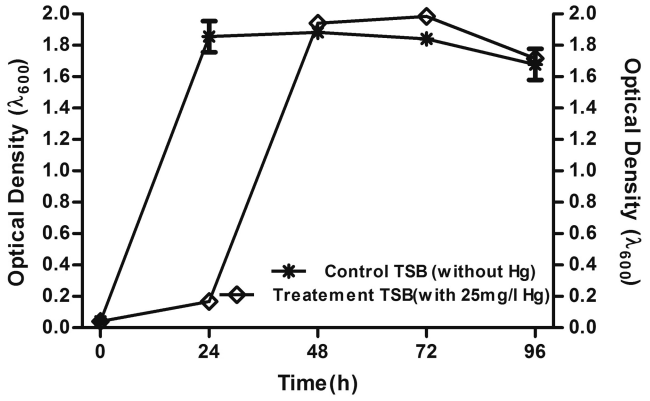

Fig. 1. Comparative growth profile of RS-5 with mercury (25 mg/l) and control in TSB.

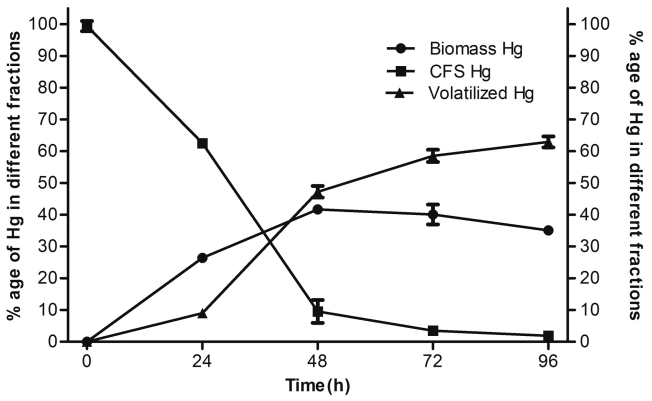

Fig. 2. Percentage of mercury in biomass, cell-free supernatant, and trapping solution after 96 h.

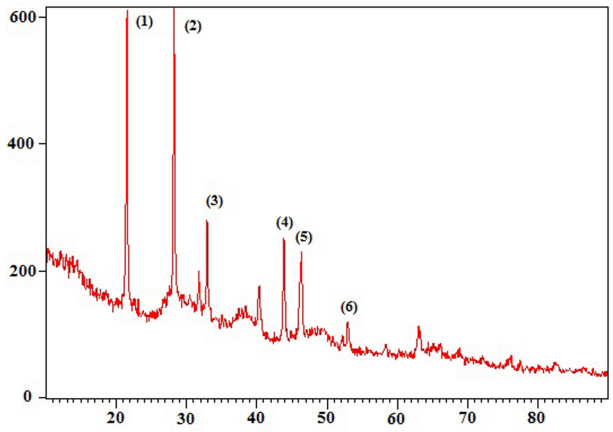

Fig. 3. X-ray diffraction pattern of biomass-associated mercury.

Characterization and Identification of RS-5

RS-5 was characterized in terms of its morphological, physiological, and biochemical properties. As such, RS-5 is rod-shaped, Gram-positive, motile, and a spore former, and has swollen sporangia. The strain develops capsule and non-pigmented colonies. Physiologically, it can tolerate a wide range of pH values (5.5–9.0), a temperature range of 15–45°C, and a high salt concentration [7% (w/v)]. The culture can be maintained and grown under aerobic conditions, is catalase and oxidase positive, does not reduce nitrate, and hence is considered an obligate aerobic organism (Table 1). Analysis of the 16S rRNA gene sequence by a multiple sequence alignment (BLAST) indicated a 99% homology to *Lysinibacillus* with a 98% alignment coverage over 1.5 kb. The sequence was assigned the GenBank accession number HM179549. The classification of RS-5, as belonging to the genus *Lysinibacillu*s, was confirmed using the RDP II Classifier [21]. For the phylogenetic profiling, additional related sequences of other bacteria were obtained from the GenBank and Ribosomal Database Project (RDP II release 9.58) [3, 5]. The sequences were aligned using Clustal W [18] and the phylogenetic analysis carried out using MEGA 4.0.1 [8] based on generating a boot-strap-corrected maximum parsimonious tree (Fig. 4).

Discussion

The major long-term aim behind this research is to exploit microorganisms for the *in situ* sequestration and transformation of mercury from industrial effluents, thereby lowering the mercury discharge into water bodies. Therefore, bacterial strains were isolated from industrial effluents and their mechanism(s) of mercury transformation explored. As a result, the reported mercury-tolerant bacterial isolate with multiple metal resistances can be exploited in managing heavy metal pollution. The efficient sequestration of divalent

Table 1. Morphological, physiological, and biochemical characteristics of RS-5.

Characteristics	Observations	Characteristics	Observations
Morphological characteristics		Biochemical characteristics	
Cell shape	Rods	Indole utilization	-ve
Cell size	0.3 µm × 1.2 µm	Methyl Red	-ve
Gram stain	Gram +ve	Voges–Proskeur	-ve
Motility	+ve (peritrichous)	Citrate utilization	+ve
Capsule	+ve	Nitrate reduction	-ve
Colony morphology on NA plates	White opaque	H_2S production	-ve
Pigmentation	No pigment	Urease production	-ve
		Starch hydrolysis	-ve
Physiological characteristics		Casein hydrolysis	-ve
Growth at 4°C	-ve	Lipid hydrolysis	-ve
Growth at 42°C	+ve	Gelatin liquefaction	+ve
Growth at 45°C	-ve	Oxidase	+ve
Growth with 1% NaCl	+ve	Catalase	+ve
Growth with 5% NaCl	+ve	Carbohydrate fermentation test	
Growth with 7% NaCl	+ve	Sucrose	-ve
Growth with 10% NaCl	-ve	Glucose	-ve
Growth at pH 2	-ve	Lactose	-ve
Growth at pH 5	-ve		
Growth at pH 8	+ve		
Growth at pH 11	-ve		

NA, nutrient agar.

Table 2. Crystallographic characterization of biomass-associated mercury.

Sr. No.	Biomass sample			Reference sample[a]		
	Pos. [°2Th.]	d-Spacing [Å]	Rel. Int. [%]	Pos. [°2Th.]	d-Spacing [Å]	Rel. Int. [%]
1	21.4212	4.14	97.87	21.4212	4.14	97.87
2	28.1691	3.16	100.00	28.1691	3.16	100.0
3	32.8311	2.72	26.65	32.8311	2.72	26.65
4	43.8051	2.06	30.39	43.8051	2.06	30.39
5	46.2964	1.96	18.06	46.0600	1.96	18.06
6	52.9458	1.72	5.17	52.8258	1.73	5.17

[a]Calos *et al*. [4].

mercury, followed by its reduction and volatilization with the intermittent formation of elemental mercury, will help facilitate the immobilization of mercury in the biomass and its deposition in far away areas, respectively.

Studies on the sequestration and reduction of mercury by bacteria have already been reported in relation to a diverse variety of bacterial genera, such as *Bacillus*, *Vibrio*, Coryneform bacteria, *Cytophaga*, *Flavobacterium*, *Achromobacter*, *Alcaligenes*, *Micrococcus* sp., *Serratia marcesens* [11], *Citrobacter* sp., and Cyanobacteria, including *Limnothrix planctonica*, *Synechococcus leopoldiensis,* and *Phormidium limnetica* [13]. However, there is limited available data on the volatilization of mercury by bacteria [9]. The isolate RS-5 was able to grow with significantly higher concentrations of mercury than those reported in industrial effluents, plus it exhibited the ability to sequester and reduce the mercury inside the biomass. Although the initial growth in the presence of mercuric ions was relatively slow, the bacteria retrieved its normal growth later on and transformed almost 97% of the mercury inside the cellular biomass. A significant fraction (63%) of the mercury was also found in the trapping solution, proving the volatilization of the mercury. An XRD analysis of the biomass grown in the mercury-supplemented media confirmed the presence of mercurous chloride in the biomass, representing an effective reduction mechanism by RS-5 to mitigate the availability of divalent mercury. In brief, it would appear that the mercuric chloride was first converted into elemental mercury and then combined with the residual intracellular mercuric chloride, resulting in calomel (HgCl)

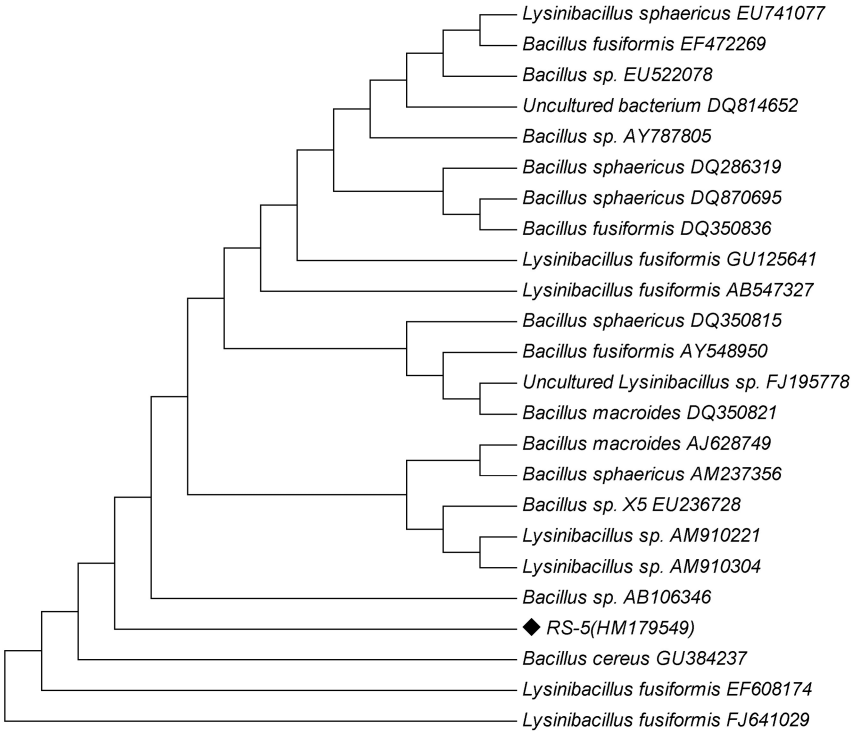

Fig. 4. Phylogenetic tree of *Lysinibacillus* sp. with other known and sequenced *Lysinibacillus* strains based on the 16S rRNA gene sequence using maximum parsimony analysis.

formation, a monovalent form of mercury. Over the 96 h experimental period, the total presence of mercury in the various fractions (*viz.*, the cell-free supernatant, biomass, and trapping solution) indicated that the organism diverted the excess sequestered mercury by volatilization and conversion of the mercuric chloride into mercurous chloride. This substantial volatilization and reduction of mercuric chloride revealed an inherited and indigenous property of the strain towards the transformation of mercuric ions to a non-available and volatilized form.

The present study isolated a potential mercury-tolerant bacteria from industrial effluent that exhibited efficient sequestration, reduction, and volatilization of mercuric ions. The present results favor exploiting microorganisms for the sequestration of heavy metals within a biomass and/or their deposition in far away areas after volatilization. This may provide a useful approach for remediating mercury-rich industrial effluents. Interestingly, this is the first report on the reduction of divalent mercury into monovalent mercury, along with the sequestration and volatilization of mercury by *Lysinibacillus fusiformis*.

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