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Characterization and Identification of A Sludge-Associated Bacterial Isolate

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ABSTRACT

During a metal speciation study, an unusually high retention of lead was observed when lead solution was percolated through a column packed with sludge compost obtained from Hampton Roads Sanitation department. A bacterium was isolated from the sludge compost and identified as *Bacillus sphaericus* using electron microscopy, whole cell fatty acid analysis (Midi System) and Biolog GP Microplate. The isolate grows in broth and agar media containing up to 800 μM lead. Lead accumulation study using atomic absorption spectrophotometer indicates that the isolate adsorbs lead. Lead adsorption is pH dependent. The isolate contains a plasmid of approximately 40 – 50 kbp that might be involved in resistance to lead. Studies are in progress to characterize this plasmid. The bacterial isolate has the potential of being used in bioremediation of heavy metal contaminated water and could be involved in localized accumulation of lead in gardens if heavy metal containing sludge compost is used as fertilizer.

INTRODUCTION

Composted wastewater sludge serves as sources of nutrients when applied to lawns and gardens. This method of disposal of wastewater sludge is currently preferred to incineration and landfilling. There is variation in bacterial composition of sludge. Some of the most frequently found bacteria in sewage are in the genera *Beggiatoa, Achromobacter, Sphaerotilus, Flavobacterium, Pseudomonas, Escherichia, Enterobacter* and *Zooglea*. However, this bacterial composition changes during sewage treatment and when sludges obtained from sewage treatment plants are used in compost production. Temperature generated during composting results in the displacement of mesophilic bacteria by thermophilic bacteria. The latter group includes species of *Clostridium, Thermoactinomycetes* and *Bacillus*. Studies have shown that wastewater sludge contain high concentrations of cations. A study of the residual metal contents of sludge from Hampton Roads in Virginia revealed the presence of copper, iron, manganese, lead and zinc. Thus, sludge-composts are potential sources of bacterial and heavy metals contamination of water and soil.

The roles of microorganisms in heavy metal uptake are subjects of many studies. These studies include the uptake of Mn, Sr, Zn, Cu and Cd by *Saccharomyces cerevisiae* (Avery and Towbin, 1993); accumulation of cesium from soil by two

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Rhodococcus sp. (Tomioka et al., 1994); accumulation of nickel and zinc by filamentous bacterium in the genus *Thiothrix* (Shuttleworth and Unz, 1993); accumulation of copper by yeasts (Junghans and Straube, 1991). Studies conducted by Mullen et al. (1989) showed that *Bacillus subtillis*, *B. cereus*, *Escherichia coli* and *Pseudomonas aeruginosa* were able to remove one or more of a variety of cations from solutions. Biosorption of metals by microorganisms occurs by different mechanisms and is affected by different factors. Metabolism-independent biosorption in some microorganisms occurs on the cell wall and capsule (Shuttleworth and Unz, 1993; Dyer et. al., 1994; Avery and Tobin, 1993; Ortega-Calva et al., 1994; Gadd, 1990; Scott and Palmer, 1988). According to Silver and Walderhaug (1992), genes located on bacterial chromosomes are responsible for bacterial accumulation of several cations and anions. The group also showed that resistances to some heavy metals are plasmid-mediated. Presence of other ions may also influence metal uptake by some microorganisms. As was reported by Macaskie et al. (1994), accumulation of heavy metals by *Citrobacter* sp. was mediated by the presence of phosphate in the periplasmic space. Drastic increase in mercuric chloride uptake by genetically engineered Escherichia coli was observed in the presence of Na (Selifonova and Barkay, 1994). These show that bacteria introduced into the soil could promote localized concentration of heavy metals, which could have serious consequences on agriculture. Also that bacteria have great potential in bioremediation of heavy metal contaminated environments.

During the course of metal speciation studies conducted using a column packed with sludge compost, an unusually high retention of lead by the column was observed when a lead solution was percolated through the column. Bacteriological examination of the compost revealed the presence of a bacterium, which was isolated and characterized. In this report, we describe the characterization and identification of a bacterial isolate from composted wastewater sludge from Hampton Roads in Virginia.

MATERIALS AND METHODS

Isolation and Growth of Bacterium. Composted sludge was sifted to remove large organic debris. This was suspended in sterile, phosphate buffered saline, pH 7.2 (PBS) and mixed thoroughly. The suspension obtained was used as inoculum for tryptic soy broth, which was incubated overnight at room temperature. The broth culture was streaked on tryptic soy agar plates and incubated. Bacterial pure culture obtained was stored on tryptic soy agar slant and at -70 C in glycerol (20% v/v in tryptic soy broth).

Bacterial Plasmid. Tryptic soy broth containing 400 μM lead nitrate was inoculated with a single colony of the isolate and incubated at 30 C. in an orbital shaker incubator. Plasmids were extracted using the rapid alkaline lysis method as described by Birnboim and Doly (1979) with slight modification. Plasmid was digested with restriction endonucleases and analyzed on a 0.6% agarose gel prepared in Tris Acetate EDTA buffer. Gel was stained in ethidium bromide (0.5 mg/L), destained and bands were visualized using the Bio-Rad Gel Doc 1000.

Physical and Biochemical Characterization. The following tests were performed on the isolate using standard bacteriological procedures: Gram's stain, growth in different concentrations of NaCl, growth at different pH and temperatures, starch hydrolysis, DNA hydrolysis, citrate utilization, catalase production, indole production, methyl red (MR) and Voges Proskauer (VP).

Electron Microscopy. Transmission electron microscopy was done on 24 and 48 hr cultures of the isolate. The isolate was fixed in 2% osmium tetroxide in 0.2M cacodylate buffer, dehydrated in a series of increasing concentration of ethanol, embedded in epon and sectioned using an LKB Ultrotome III 8800. Electron microscopy was done using the JEOL 100CX II Transmision Electron Microscope.

Whole Cell Fatty Acid Analysis. Fatty acid analysis was performed using the MIDI system. Bacterial isolate was inoculated on Tryptic Soy Broth Agar (TSBA) in petri dishes and incubated at 29° C. Approximately 35mg of the cells were harvested into a precleaned, screw-capped tube. The cell pellet was treated as specified by the manufacture in order to extract cell-associated fatty acids. The extracted fatty acids were resolved using a HP 5890 gas chromatograph and Sherlock Pattern Recognition Software.

Biolog GP Microplate Analysis. The Biolog Gram Positive Microplate method using 95 biochemical tests was done according to the manufacturer's direction. The GP Microplate determines the ability of bacterial isolates to oxidize 95 different carbon sources (Figure 1). The isolate was grown in Biolog Universal Medium containing glucose. Bacterial suspension was made in physiological saline and 150 µL of the suspension was inoculated into microtiter plate wells containing different carbon sources and also tetrazolium dye. The microtiter plate was incubated at 30° C for 24 hrs. When the carbon source is utilized there is a blue color formation resulting from reduction of tetrazolium dye incorporated into the medium. Negative wells remain colorless. The result yields a metabolic fingerprint of the inoculated organism

which is entered into Biolog's Microlog computer Identification of isolate is by computerized-comparison of the results with metabolic pattern of library species kept in database.

Lead Accumulation Studies. SB-1 was inoculated into LB broth and incubated overnight at 26° C in a shaker incubator. The culture was centrifuged, the pellet washed in sterile deionized water and suspended in water to a known absorbance. This suspension was used as inoculum for screw-capped tubes containing equal volume of physiological saline supplemented with varying concentrations of the metal under study. The tubes were incubated at 26° C in a shaker incubator and samples were retrieved at set intervals of time. The retrieved samples were centrifuged. The supernatants obtained were saved and the pellets washed with distilled water and digested in 1N HNO_3 as described by Shuttleworth and Unz (1993). The metal contents of the digested pellets and the corresponding supernatants were determined using a Perkin-Elmer 4000AAS and the appropriate Intensitron lamp.

RESULTS AND DISCUSSION

The results of several Gram's staining procedure showed that the bacterial isolate was Gram negative rod. Contrarily, transmission electron microscopy result shown in Figure 2 indicates that the isolate is a sporulating, long rod with round, subterminal endospores. These characteristics are associated with Gram positive bacteria. Old cultures of Gram positive bacteria and Gram positive bacteria with thin peptidoglycan will, in some cases, give a Gram negative result. Electron microscopy shows that the bacterial isolate (SB-1) has thin cell wall. The isolate exhibited very good growth at pH 6.0 – pH 9.0 but very little growth at pH 4.0. Copious growth was evident at up to 4.0% NaCl but very little growth at higher concentration of NaCl. The isolate is

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Water	α-cyclodextrin	β-cyclodextrin	dextrin	Glycogen	Inulin	Mannan	Tween 40	Tween 80	N-acetyl-D-Glucosamine	N-acetyl-B-D-Mannosamine	amygdalin
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
L-Arabinose	D-Arabitol	Arabutin	D-Cellobiose	D-Fructose	L-Fucose	D-Galactose	D-Galacturonic acid	Gentiobiose	D-Gluconic acid	A-D-Glucose	M-Inositol
C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
A-D-Lactose	Lactulose	Maltose	Maltotriose	D-Mannitol	D-Mannose	D-Malezitose	D-Melibiose	A-methyl-D-Galactoside	B-methyl-D-Galactoside	3-methyl glucose	A-methyl-D-galactoside
D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
B-methyl-D Glucoside	A-methyl-D-Mannoside	Palatinose	D-Psicose	D-Raffinose	L-Rhamnose	D-Ribose	Salicin	Sedoheptulosan	D-Sorbitol	Stachyose	Sucrose
E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
D-Tagatose	D-Trehalose	Turanose	Xylitol	D-Xylose	Acetic acid	A-Hydroxy-butyric acid	B-Hydroxy-butyric acid	G-hydroxy-butyric acid	P-hydroxy-phenyl acetic acid	A-keto-Glutaric acid	A-keto-Valeric acid
F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Lacamide	D-lactic acid Methyl ester	L-Lactic acid	D-Malic acid	L-Malic acid	Methyl Pyruvate	Methyl Succinate	Propionic Acid	Pyruvic Acid	Succinamic Acid	Succinic Acid	N-acetyl-L-Glutamic acid
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
Alaninamide	D-Alanine	L-Alanine	L-Alanyl Glycine	L-Asparagine	L-Glutamic Acid	Glycyl-L-Glutamic Acid	L-Pyroglutamic Acid	L-Serine	Putrescine	2,3-Butanediol	Glycerol
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
Adenosine	2-Deoxy adenosine	Inosine	Thymidine	Uridine	Adenosine-5-'Mono-phosphate	Thymidine-5'-Mono-phosphate	Uridine-5'-Monophos-phate	Fructose-6-Phosphate	Glucose-1-Phosphate	Glucose-6-Phosphate	D,L-A-Glycerol Phosphate

FIGURE 1. BIOLOG GP Microplate substrates used in SB-1 bacterial identification.

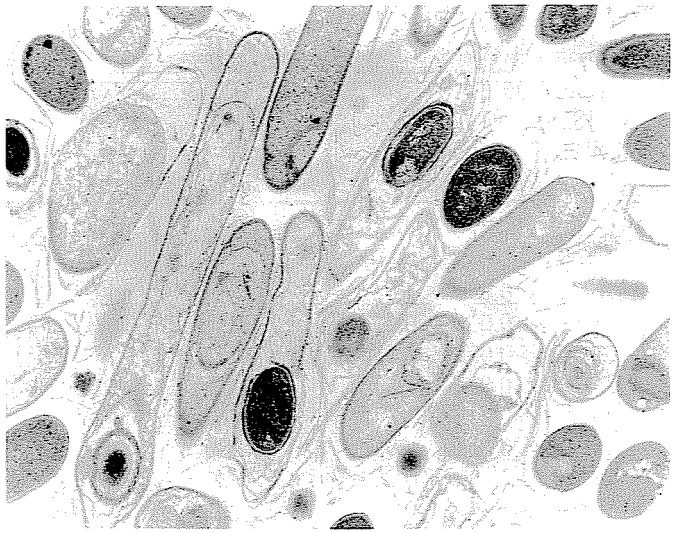

FIGURE 2. Electron micrograph of SB-1 bacterial isolate showing subterminally located endopospores in the bacilli.

amylase, oxidase and VP negative but catalase, Dnase and MR positive. Whole cell fatty acid analysis profile of the isolate is shown in Table 1. The result indicates that the isolate is Gram positive because it contains anteiso and iso fatty acids, which are found only in Gram positive bacteria. In addition, the bacterium was identified as *Bacillus sphaericus* based on whole cell fatty acid analysis. This identification was supported by the Biolog GP Microplate results. The similarity index of the Biolog GP Microplate identification was 0.892. A similarity index of at least 0.5 for cultures incubated at 30° C is acceptable for species identification.

The isolate contains a large plasmid that is approximately 40 - 50 kbp. The plasmid has EcoR I, Hind III and Hae III sites but not BamH I Sal I and Sma I sites. Although the function of this plasmid is yet to be determined, the plasmid is believed to be associated with the survival of the isolate in lead and probably other heavy metals. Plasmid preparation from culture grown in nutrient broth without lead resulted in very little yield. On the other hand, culture of the isolate in medium containing up to 40 μM lead resulted in a good yield of plasmid. According to Silver and Ji (1994) and Collard et al. (1994), resistances to heavy metal in some Gram negative bacteria reside in large, naturally occurring plasmids. Studies have begun to characterize the plasmid.

Initial studies indicated that the isolate is capable of growing in culture medium containing up to 600 μM lead, this indicates resistance to lead because lead at very small concentrations is detrimental to bacterial growth. In their studies of bacterial communities in heavy metal contaminated soils, Roane and Kellogg (1996) isolated many lead-resistant genera including *Bacillus*. The mechanism of resistance to the heavy metals was not discussed. The isolate also accumulates lead from solution (Figure 3). Lead accumulation increases with increase in the cell concentration and was pH dependent. Lead accumulation was highest at pH 7.3 and pH 8.0 than at pH 5.0. This finding is supported by Tomioka et al (1994) working with *Rhodococcus* species. They showed that maximum absorption of cesium occurred at pH 8.5. The mode of

TABLE 1. Fatty acid analysis and identification of SB-1 bacterial isolate.

Sherlock Version: 1.06
ID: 17651 AA-SB-960712
Bottle:27 SAMPLE (AEROBE)

RT	Area	Ar/Ht	Respon	ECL	Name	%
1.667	252780000	0.026	----	7.013	SOLVENT PEAK	---
2.828	102	0.027	----	9.543	----------	---
6.367	7974	0.035	1.012	13.618	14:0 ISO	8.62
6.858	768	0.035	0.997	14.000	14:0	0.82
7.769	27366	0.039	0.974	14.621	15:0 ISO	28.46
7.901	13374	0.039	0.970	14.711	15:0 ANTEISO	13.86
8.325	648	0.039	0.961	15.000	15:0	0.67
8.943	11766	0.041	0.949	15.386	16:1 w7c alcohol	11.93
9.326	25146	0.040	0.942	15.625	16:0 ISO	25.30
9.538	3348	0.040	0.938	15.758	16:1 wlle	3.36
9.926	1692	0.044	0.932	16.000	16:0	1.68
10.729	756	0.041	0.920	16.478	Sum In Feature 5	0.74
10.983	1626	0.043	0.917	16.629	17:0 ISO	1.59
11.141	3036	0.044	0.915	16.723	17:0 ANTEISO	2.97
19.600	10008	0.078	----	21.652	----------	---
*****	756	----	----	----	Summed feature 5	.74

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref
252780000	79602	97500	99.90	93613	9

TSBA [Rev 3.90] Bacillus 0.410
B. sphaericus 0.410
B. s. GC subgroup IV* . . . 0.410
B. s. GC subgroup III . . . 0.376
CLIN [Rev 3,90] * NO MATCH *

lead biosorption is most likely by adsorption to the cell wall because the lead accumulation studies were conducted using cells suspended in physiological saline. Several studies including that of Strandberg et al., (1981) have shown that bacteria and other microorganisms with cell wall adsorb heavy metals to their cell wall. In a review article, Gadd (1988) indicated that biosorption of heavy metal by *Bacillus subtilis* occurs on the carboxyl group of glutamic acid component of the peptidoglycan. Nakajima and Sakaguchi (1986) showed that *Bacillus subtilis* exhibited extremely high absorption of heavy metals, including lead, copper, uranium, cobalt and nickel. Gram negative bacteria have been the foci of studies for bacterial bioremediation abilities. This study shows that non-pathogenic *Bacillus* species may be important in bioremediation of heavy metal contaminated soil and aquatic environments.

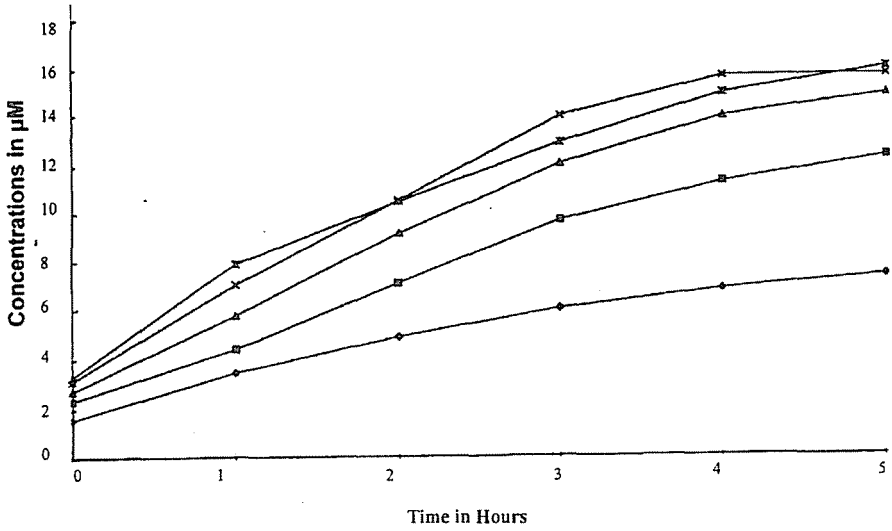

Figure 3. Lead concentrations in SB-1 pellets from solutions containing various lead concentrations (♦, ■, ▲, X and * represent 10, 20, 40, 80 and 100 mM lead, respectively).

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