JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Nov 2015. Vol. **9**(Spl. Edn. 2), p. 113-118

Leucobacter sp., A Heavy Metal Resistant Bacterium Isolated from Paper Mill Solid Effluent

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(Received: 20 July 2015; accepted: 10 October 2015)

A Gram-positive, rod-shaped, aerobic bacterial strain isolated from the polluted soil of paper mill contaminated with various heavy metals. The isolated strain was tested for their resistance to different heavy metals (Ni, Cu, Zn and Cd) in various concentrations of (0.1, 0.5, 2.0, 4.0 mM). The relative growths (%) at 2mM concentration were observed as Cu (49.48%)> Ni (40.8%)>Cd (15.33%) > Zn(13.93 %). The heavy metal resistant in the isolated strain was found to be Cu> Ni>Cd>Zn at higher concentrations. The strain showed positive activity towards catalase, starch amylase, Lysine utilization, H_2S production and citrate utilization and showed negative activity against ONPG, Urease activity, nitrate reduction, Voges Proskauer's (VP) test, methyl red and oxidase production. The strain (KC602304) appeared to be most susceptible being inhibited by majority of antibiotics and found resistant towards Ampicillin. In silico study was conducted to understand the major evolutionary relationship among the different species of *Leucobacter* species of nucleotide sequence of 16s ribosomal RNA with the isolated strain (KC602304) of *Leucobacter* sp. obtained from Gene Bank.

Key words: Heavy metals, Gram (+) bacteria, in silico, 16SrRNA, Gene Bank.

Heavy metals are released in to soils from industrial operations such as mining, manufacturing of alkaline storage batteries, combustion of fossil fuel [1, 2]. The paper mill solid effluents have been found to contain approximately 700 organic and inorganic compounds and are classified as carcinogenic and mutagenic compounds[3]. Heavy metals are non-biodegradable and are deposited and absorbed into the tissues of plants and animals and at higher concentrations, the heavy metals become toxic thus affecting the growth, morphology, metabolic activities of the soil microorganisms[4,5]. Gram positive bacteria are found to have high metal sorption capacity by the cell walls and tolerate high concentrations of heavy metals[6]. Gram positive bacteria are found to tolerant to various heavy metal stresss by intra and extra cellular sequestration, active transport, efflux pumps, enzymatic detoxification, and reduction in the sensitivity of the cellular targets to metal ions[7, 8]. Metal resistance physiology in sixty three species of bacteria were examined with the protein-level similarities and observed that these metal resistant bacteria can be developed into metal pollution biosensors[9]. Bacterial siderophores play an important role in heavy metal tolerance for protecting bacteria against heavy metal toxicity[10].

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The genus *Leucobacter* gram (+) positive, aerobic, rod-shaped and was first proposed by Takeuchi *et al*[11]. A number of chromate resistance *Leucobacter* sp. have been reported from activated sludge[12-18].

MATERIALS AND METHODS

Description of the study area

The present study was conducted at Panchgram,Hailakandi,Assam,adjoining the cachar Paper Mill an unit of Hidustan paper corporation limited a Government of India undertaking. Geographically the site is situated at longitude of 24°41'29.9"N and latitude at 92°45'25.9"E' with an altitude of about 36 m above MSL.

Isolation and chracterization of bacterial isolate

For bacterial isolation, soil sample (1g) was suspended in 10ml of distilled water and serial dilutions were spread on starch-caesin agar. (1% soluble starch, 0.03%casein, 0.2% KNO_3, 0.2% NaCl, 0.2% KH_2PO_4, 0.002%$CaCO_3$, 0.005% $MgSO_4$.$7H_2O$, 0.001% Fe SO_4, $7H_2O$, 1.8% agar and pH 7.2). Inoculated plates were incubated at30°C for 7 days. The isolate was preserved in a 20% (v/v) glycerol suspension at -30°C.

Morphologiacal and Biochemical characterization of bacterial isolate

The cultural and morphological features falls under the phenotypic characterization, which were studied by adopting standard methods[19]. Gram staining was done by Gram Stains kit (Himedia K001).For biochemical characterization, the isolate was tested for ONPG, Lysine utilization, ornithine utilization, urease activity, Phenylalanine deamination, nitrate reduction, H_2S production, citrate utilization, Voges-Proskauer test, Methyl red test, Indole production, Malonate utilization, Oxidase production, Starch amylase test, Catalase activity etc. Fermentative degradation of various carbohydrates (Glucose, sucrose, xylose, maltose, rhamnose, raffinose, cellubiose, dextrose, gallactose, arabinose, lactose, sorbitol,melibiose, saccarose and trehalose) an indicator (phenol red) and pH-7.3. Extraction of genomic DNA, PCR amplification and sequencing of the16S rRNA gene were carried out as described by Greg[20]. Identification of the bacterial isolate was carried out according to Bergey's Manual of Systematic Bacteriology[21].

Determination of antibiotic resistance

The isolate was tested for antibiotic sensitivity according to Kirby-Bauer disc diffusion method to 12 antibiotics[22]. Discs containing the following antibiotics were used as Penicillin G (10 units), Polymyxin B (300 units), Streptomycin (10 mcg), Vancomycin (30 mcg), Tetracycline (30mcg), Gentamycin (120mcg), Rifamycin (5mcg), Amikacin (30mcg), Ampicillin (10mcg), Chloramphenicol (30mcg), Ciprofloxacin(10mcg) and Levofloxacin (10mcg).

Evaluation of metal resistant bacteria

The selected bacterial isolate was tested for their resistance to different heavy metals by their growth in nutrient broth tubes containing various concentrations of heavy metals (0.1, 0.5, 2.0, 4.0 mM). The metals selected for the present investigation included Ni, Cu, Zn and Cd. These tubes were inoculated with freshly grown culture of the isolate and incubated at 30± 2.0°C for 48 h. The bacterial growth was determined by measuring the optical density using spectrophotometer at 540 nm. Relative growth of the isolate was expressed as the percentage of those obtained in untreated control.

Identification of metal resistant bacteria

The isolation and purification of chromosomal DNA as well as the amplification and sequencing of partial 16S rRNA gene of potential metal resistant bacteria isolate was carried out. The nucleotide sequence of bacterial isolate thus obtained was compared for sequence similarity level with the reference species of bacteria contained in genomic database using the "NCBI BLAST"[23].

Genotypic characterization of isolated bacterial strain

Phylogenetic and molecular evolutionary analyses of the isolate was conducted using software MEGA version 4.0 package[24]. The 16S rRNA gene sequences of the potential metal resistant bacteria isolate was aligned using the CLUSTAL W program against corresponding nucleotide sequences retrieved from Genbank database[25]. A phylogenetic tree was constructed using the neighbour-joining (NJ) method and by NCBI on-line service which showed the relationships with their closely related neighbouring species[26]. The sequence of the isolated strain in this study was deposited and

accession number (KC602304) was obtained from Gene Bank.

RESULTS AND DISCUSSION

The isolated strain of *Leucobacter* sp. showed resistant to higher concentrations of Ni, Cu, Zn and Cd. The relative growths (%) of *Leucobacter* sp. (KC602304) at 2mM concentration was found to be Cu (49.48%)> Ni (40.8%)>Cd (15.33%) > Zn(13.93 %).At higher concentrations of heavy metals (4mM), the relative growths (%) of *Leucobacter* sp. (KC602304) was found to be Cu (11.97%)> Ni (10.16%)>Cd (9.64%) > Zn(9.40 %) (Table1). Thus, the heavy metal resistant in the strain was found to be Cu>Ni>Cd>Zn.

The isolated strain of *Leucobacter* sp. showed positive activity towards catalase, starch amylase, Lysine utilization,H_2S production and citrate utilization and showed negative activity against ONPG, Urease activity, nitrate reduction, Voges Proskauer's (VP) test, methyl red and oxidase production (Table 2). The isolated strain showed positive for the production of acids against various sugars tested like Glucose, Sucrose, Maltose, Rhamnose, Rafffinose, Cellubiose, Gallactose, Lactose, Sorbitol and negative against Xylose, Rhamnose, Dextrose,Arabinose, Melibiose, Saccarose, Trehalose (Table 3). The strain (KC602304) appeared to be most susceptible being inhibited by majority of antibiotics and found resistant towards Ampicillin (Table 4).

The isolate was found to be resistant at higher concentrations of copper and Nickel and relative growth of (40-50) % observed at higher concentrations of heavy metals. It showed positive against biochemical tests i.e., catalase, starch amylase, Lysine utilization, H_2S production and citrate utilization. Similar chemotaxonomic characteristics were observed earlier by various workers[27, 13 and 18]. The bisorption of Nickel and reduction of chromium by *Leucobacter* sp. was observed by various workers who reported choromate transport protein A(ChrA) that has the metal tolerance capacity by chromate ion efflux. 16srRNA gene phylogenetic analysis also suggested that the isolated bacterial strain (KC602304) from polluted soil closely related to *Leucobacter* sp[28, 17] (Fig. 1). Our results are also in conformity with the results of various workers who described the *Leucobacter* strain has the ability to tolerate heavy metal stress by metal ions

Table 1. Relative growth (%) of bacterial isolate in nutrient broth containing different heavy metals.

Metal tested	Incuabation period	Heavy metal (mM) concentration	Relative growth (%) of bacterial strain (KC602304)
Ni	48		
		0.1	86.40
		0.5	68.07
		2.0	40.8
		4.0	10.16
Cu	48	0.1	93.43
		0.5	80.29
		2.0	49.48
		4.0	11.97
Zn	48	0.1	92.30
		0.5	78.71
		2.0	13.93
		4.0	9.40
Cd	48	0.1	50.93
		0.5	22.44
		2.0	15.53
		4.0	9.64

Each value represents average of duplicates

Table 2. Morphological and biochemical characteristics of isolated bacterial strain

SL No.	Morphological and Biochemical test	KC602304
1.	Gram staining	+
2.	ONPG	-
3.	Lysine utilization	+
4.	Ornithine utilization	+
5.	Urease	-
6.	Phenylalaninedeamination	-
7.	Nitrate reduction	-
8.	H_2S production	+
9.	Citrate Utilization	+
10.	Voges Proskauer's	-
11.	Methyl red	-
12.	Indole	-
13.	Malonate utilization	+
14.	Oxidase production	-
15.	Starch amylase	+
16.	Catalase	+

(+) = positive; (-) = negative.

Table 3. Production of acids from sugars by the isolated bacterial strain

Sl.No.	Sugars	KC602304
1.	Glucose	+
2.	Sucrose	+
3.	Xylose	-
4.	Maltose	+
5.	Rhamnose	-
6.	Raffinose	+
7.	Cellubiose	+
8.	Dextrose	-
9.	Gallactose	+
10.	Arabinose	-
11.	Lactose	+
12.	Sorbitol	+
13.	Melibiose	-
14.	Saccarose	-
15.	Trehalose	-

(+) = positive; (-) = negative.

Table 4. Antibiotic sensitivity profile by the isolated bacterial isolate

Sl. No.	Antibiotics disc (conc.)	KC602304
1.	Penicillin G (10 units)	34(S)
2.	Polymyxin B (300 units)	20(S)
3.	Streptomycin (10mcg)	31(S)
4.	Vancomycin (30 mcg)	26(S)
5.	Tetracycline (30mcg)	35(S)
6.	Gentamycine (10 mcg)	36(S)
7.	Rifamycin (30 mcg)	35(S)
8.	Amikacin (30mcg)	34(S)
9.	Ampicillin (10 mcg)	10(R)
10.	Chloramphenicol (30 mcg)	28(S)
11.	Ciprofloxacin(10mcg)	34(S)
12.	Levofloxacin(10mcg)	36(S)

NI = No Inhibition; Diameter of disc =6mm;R = Resistant; I = Intermediate; S = Susceptible

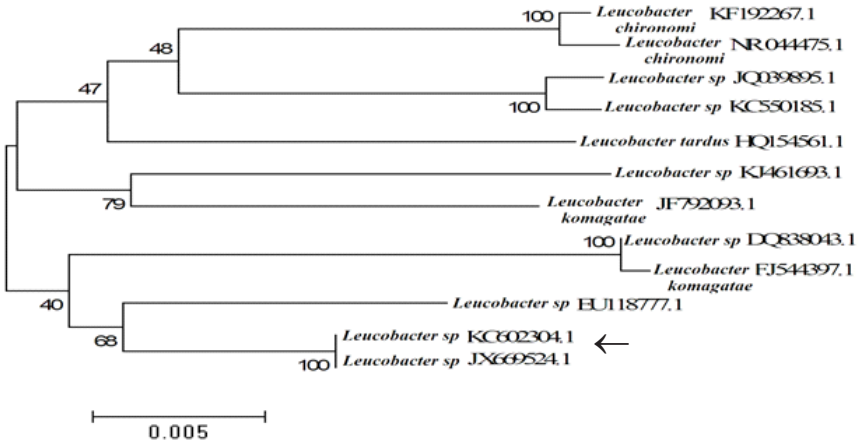

Fig. 1. Neighbour-joining tree of 16SrRNA gene sequences from isolate of *Leucobacter* sp. (KC602304) with 16SrRNA of other bacteria obtained from gene bank. The Kimura two-parameter substitution model was used and the nodes are supported by 1,000 bootstrap replications. Bootstrap values above 50% and the genetic distance scale are shown (Mega 4.1 version).

transport across the cell membrane, biosorption to cell walls and entrapment in extracellular capsules, precipitation, complexation and oxidation-reduction reaction[29,30]. The present study interpreted that the isolated strain of *Leucobacter* sp. is tolerant to heavy metals Cu> Ni>Cd>Zn at higher concentrations and has great potential to be used as a bioremediation agent in the contaminated soils of paper mill by alleviating the metal toxicity and supplying the plant with nutrients.

CONCLUSION

From the present study, it can be concluded that the isolated strain of *Leucobacter* sp. is able to tolerate higher concentrations of heavy metals. It may be termed as the isolated strain

of *Leucobacter* sp. (KC602304) as heavy metal tolerant strain by its phenotypic and genotypic features which might be utilized as potential bioremediation agent in paper mill polluted soil contaminated with various heavy metals.

ACKNOWLEDGEMENTS

The authors are grateful to the common wealth scholarship grant, Canada for carrying out the present work in the Department of Biological Science, University De Montreal, Montreal, Canada for providing the laboratory facilities.

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