Research Paper

Volume : 3 | Issue : 12 | December 2014 • ISSN No 2277 - 8179

Isolation and characterization of Chitinophaga sp. from paper mill polluted soil.

Microbiology

KEYWORDS : Heavy metal, Gram (-) bacteria, in silico, 16SrRNA, Gene Bank.

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ABSTRACT

Two Gram-negative, rod-shaped, deep yellow-pigmented bacteria were isolated from the polluted soil of paper mill contaminated with various heavy metals. The strains were tested for their resistance to different heavy metals (Ni,Cu, Zn and Cd) by their growth in nutrient broth tubes containing various concentrations of (0.1, 0.5, 2.0, 4.0 mM). The relative growths (%) at 2mM concentration were observed as (18.70 and 17.08 %) in Ni, (13.71and 15.81%) in Cu, (18.63 and22.37%) in Zn and (18.36 and 8.39) in Cd. The heavy metal resistant in the two strains were found to be Zn>Ni>Cu> Cd at higher concentrations. The strains showed positive activity towards urease, nitrate, H2S production, citrate utilization, methyl red, Malonate utilization, oxidase production, starch amylase and showed negative activity against ONPG, phynylalanine, Lysine utilization and Voges Proskauer's (VP) test and catalase activity. The strains were found susceptible to various antibiotics Vancomycin, Streptomycin, Amikacin, Ciprofloxacin. In silico study was conducted to understand the major evolutionary relationship among the different species of Chitinophaga species of nucleotide sequence of 16s ribosomal RNA with the isolated strains (KC602266 and KC602269) of Chitionophaga sp obtained from Gene Bank.

INTRODUCTION:

Among various pollutants, heavy metals are released in to soils from industrial operations such as mining, manufacturing of alkaline storage batteries, combustion of fossil fuel (Khan *et al*., 2000 and Kumar *et al*., 2011). Paper pulp industries are the sixth largest effluent generating industries of the world (Ugurlu *et al*, 2007). These effluents have been found to contain approximately 700 organic and inorganic compounds and are classified as carcinogenic and mutagenic compounds (Karrash *et al*., 2006). Some heavy metals are essential for the growth of microorganisms but at higher concentrations ,the heavy metals become toxic to the microorganisms thus affecting the growth,morphology, metabolic activities (Abou-Shanab *et al*., 2007;Issazadeh et al., 2013) Bacteria are found to develop five important mechanisms to detoxify the heavy metals available in contaminated soils:1) exheavyllular detoxification, 2)exheavyllular sequestration, 3)reduced permeability,4) inheavyllular sequestration and 5) export. These resistant mechanisms are encoded in bacterial plasmids and transposes due to spontaneous mutation and gene transfer (Osborn *et al*., 1997). Nies (1999) compared the metal resistance physiology in sixty three species of bacteria and examined the protein-level similarities and suggested that these metal resistant bacteria can be developed into metal pollution biosensors. Bacterial siderophores play an important role in heavy metal tolerance for protecting bacteria against heavy metal toxicity (Schalk *et al*., 2011). Long et al. (2012) described the importance efflux transporters as a metal tolerance lactic by bacteria.

The genus *Chitinophaga*, is a Gram (-) negative bacteria and first described by Sangkhobol and Skerman (1981), is the type genus of the family Chitinophagaceae (Kampfer *et al*., 2006).Lee et al. (2007) and Kim and Jung (2007) described some novel species like Chitinophaga ginsengisegetis and Chitinophaga ginsengisoli. Yasir et al.(2011) isolated *Chitinophaga eiseniae* sp. nov from vermicompost samples prepared from paper mill and dairy sludge.

MATERIALS AND METHODS:

1.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. The paper mill is surrounded by a vast spread of agriculture land used for paddy cultivation on its eastern side while the northern side has hill areas covered with dense forest. 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.

2. Isolation and chracterization of bacterial isolates

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% $KH2PO_4$, 0.002%$CaCO_3$, 0.005% $MgSO_4$ .7H2O, 0.001% Fe SO4 , 7H2O,1.8% agar and pH 7.2). Inoculated plates were incubated at300C for 7 days. The isolate was preserved in a 20% (v/v) glycerol suspension at -30^{0}C.

3. Cultural and morphological features of the bacteria isolates

The cultural and morphological features falls under the phenotypic characterization, which were studied by adopting standard methods (Rath and Subramanyam,1998).Morphology of colonies on plates is a characteristic feature of bacterium, which is quite useful in preliminary identification procedure. Different colony features such as configuration, elevation, margin, texture, consistency etc. were noted down by using a hand lens. Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Greg (2010).

4. Morphologiacal and Biochemical characterization of bacterial isolates

Gram staining, a differential staining technique separates bacteria into two groups, Gram positive and Gram negative. The test was done by Gram Stains -kit (Himedia K001). For biochemical characterization, the isolates were 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. and fermentation of fifteen different sugars. Identification of the bacterial isolates was carried out according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1994).

5. Fermentation of carbohydrates

Fermentative degradation of various carbohydrates such as glucose, sucrose, lactose, maltose, fructose, galactose, arabinose and mannitol was carried out in a fermentation tube that contained Durham's tube a small tube placed in an inverted position inside the culture tube) for the detection of gas production. The medium used contained ingredients of nutrient broth, a carbohydrate source (Glucose, sucrose, xylose, maltose, rhamnose, raffinose ,cellubiose, dextrose, gallactose, arabinose, lactose, sorbitol,melibiose, saccarose and trehalose) an indicator (phenol red) and pH-7.3. The broth was prepared separately for each set of carbon source; cultures were inoculated and incubated at 30^0C. Change of colour of the broth from red to yellow indicated the production of organic acid as a result of fermentation of the particular carbohydrate. Phenol red which was red at neutral pH (7.0) turned yellow at or below a pH of 6.8 due to accumulation of organic acid. No change in the colour when compared to the uninoculated control indicated negative for acid production.

6. Determination of antibiotic resistance

The isolates were tested for antibiotic sensitivity according to Kirby-Bauer disc diffusion method (Bauer *et al.* 1966) to 12 antibiotics The diameter of the inhibition zones was measured to the nearest mm and the isolates were classified as resistant (R), intermediate (I) and susceptible (S) following the standard antibiotic disk sensitivity testing method. Discs containing the following antibiotics were used: 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).

7. Evaluation of metal resistant bacteria

The selected bacterial isolates were 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 isolates and incubated at 30± 2.0^0C for 48 h. The bacterial growth was determined by measuring the optical density using spectrophotometer at 540 nm. Relative growth of the isolates was expressed as the percentage of those obtained in untreated control.

8. 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 bacterial isolates was carried out. The nucleotide sequence of bacterial isolates thus obtained was compared for sequence similarity level with the reference species of bacteria contained in genomic database using the "NCBI BLAST" (Altschul *et al.*, 1990).

9. Genotypic characterization of isolated bacterial strains

Phylogenetic and molecular evolutionary analyses of the isolates were conducted using software MEGA version 4.0 (Kumar *et al.*, 2004) package. The 16S rRNA gene sequences of the potential metal resistant bacterial isolates were aligned using the CLUSTAL W program (Thompson *et al.*, 1994) against corresponding nucleotide sequences retrieved from Genbank database. A phylogenetic tree was constructed using the neighbour-joining (NJ) method (Saitou and Nei, 1987) and by NCBI on-line service which showed the relationships with their closely related neighbouring species. The sequences of the two isolated strains in this study were deposited and accession numbers (KC602266 and KC602269) were obtained from Gene Bank.

RESULTS AND DISCUSSION:

The isolated two strains of *Chitinophaga* sp. were showed resistant to higher concentrations of Ni, Cu, Zn and Cd. These strains were capable to grow at higher concentrations of heavy metals. The relative growths (%) of *Chitinophaga* sp1 (KC602266) at 2mM concentration was found to be Zn (18.63%)> Ni (18.70%)>Cu (13.71%) > Cd(18.36 %) whereas the relative growth of *Chitinophaga* sp2(KC602269) was Zn 22.37%> Ni 17.08 %> Cu15.81>Cd (8.31%) At higher concentrations of heavy metals (4mM), the relative growths (%) of *Chitinophaga* sp1 (KC602266) was found to be Zn (8.07%)> Ni (7.68%)> Cd(9.60 %)> Cu (6.18%) whereas the relative growth of *Chitinophaga* sp2(KC602269) was Zn (10.05%)> Ni (9.33 %)> Cd (5.59%)> Cu(5.55%) (Table1). Thus, the heavy metal resistant in the two strains were found to be Zn>Ni>Cu> Cd.

The isolated strain of *Chitinophaga* sp was showed positive activity towards urease, nitrate, H_2S production, citrate utilization, methyl red, Malonate utilization, oxidase production, starch amylase and showed negative activity against ONPG, phynylalanine, Lysine utilization and Voges Proskauer's (VP) test and catalase activity. The isolated strain of Chitinophaga sp was showed negative against oxidase, catalase, starch amylase and negative against catalse and oxidase tests (Table 2). The similar biochemical characteristics observed in Chitinophaga sp was observed by Lee et al.(2009) and Wang et al.(2014).The selected strains were showed positive for the production of acids against various sugars tested like Glucose, Sucrose, Xylose, Maltose, Rhamnose, Rafffinose, Cellubiose, Dextrose, Gallactose, Lactose, Sorbitol, Saccarose and negative against Melibiose, Arabinose, Trehalose (Table 3). The strains (KC602266 and KC602269) were appeared to be most susceptible being inhibited by majority of antibiotics and showed no inhibition against antibiotics Penicillin and Ampicillin (Table 4).

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

Metal tested	Incuabation period	Heavy metal concentration (mM)	Relative Growth (%) of bacterial strains with the Accession Numbers	
			KC602266	KC602269
Ni	48	0.1	86.55	93.93
		0.5	73.55	78.18
		2.0	18.70	17.08
		4.0	7.68	9.33
Cu	48	0.1	90.97	90.19
		0.5	85.41	61.24
		2.0	13.71	15.81
		4.0	6.18	5.55
Zn	48	0.1	90.41	76.88
		0.5	79.46	34.59
		2.0	18.63	22.37
		4.0	8.07	10.15
Cd	48	0.1	80.35	77.72
		0.5	64.54	26.94
		2.0	18.36	8.39
		4.0	9.60	5.59

Each value represents average of duplicates

Volume : 3 | Issue : 12 | December 2014 • ISSN No 2277 - 8179

Table 2: Morphological and biochemical characteristics of isolated bacterial strains

SL No.	Morphological and Biochemical test	KC602266	KC602269
1.	Gram staining	-	-
2.	ONPG	-	-
3.	Lysine utilization	-	-
4.	Ornithine utilization	+	+
5.	Urease	+	+
6.	Phenylalanine deamination	-	-
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	-	-

Table 3: Production of acids from carbohydrates by the isolated bacterial isolates

Sl.No.	Sugars	KC602266	KC602269
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 isolates

Sl. No.	Antibiotics disc (conc.)	KC602266	KC602269
1.	Penicillin G (10 units)	NI	NI
2.	Polymyxin B (300 units)	10(I)	13(S)
3.	Streptomycin (10mcg)	30(S)	21(S)
4.	Vancomycin (30 mcg)	20(S)	33(S)
5.	Tetracycline (30mcg)	22(S)	24(S)
6.	Gentamycine (10 mcg)	29(S)	27(S)
7.	Rifamycin (30 mcg)	13(I)	18(I)
8.	Amikacin (30mcg)	12I	20(S)
9.	Ampicillin (10 mcg)	NI	NI
10.	Chloramphenicol (30 mcg)	24(I)	24(I)
11.	Ciprofloxacin(10mcg)	33(S)	32(S)
12.	Levofloxacin(10mcg)	30(S)	29(S)

NI = No Inhibition; Diameter of disc =6mm Letter in parenthesis indicate sensitivity; R = Resistant; I = Intermediate; S = Susceptible.

The resistance of these bacterial strains towards heavy metal could be a result of the interaction between the metals and amphoteric groups such as the carboxyl and phosphoryl groups that occur within the constituent polymers of bacterial cell walls which act as if they were an open ion exchange resin. The isolated strain of *Chitinophaga* sp was showed negative against oxidase, catalase, starch amylase and negative against catalse and oxidase tests. The similar biochemical characteristics observed in *Chitinophaga* sp was observed by Lee et al.(2009) and Wang et al.(2014). The strains were found to be resistant to antibiotics and heavy metal ions. This may be due to the metal isons transport across the cell membrane, biosorption to cell walls and entrapment in exheavyllular capsules, precipitation, complexation and oxidation-reduction reaction (Nasrazadani *et al*., 2011 and Adel *et al*., 2014). Matyar et al.(2008) studied the significant proportion of antibiotic and heavy metal resistant in Gram (-) negative bacteria. Multiple heavy metal resistance determinants for Ni, Cu, Hg, Zn, Cd, Co, Cr and Pb have been isolated from plasmids (Berg *et al*., 2005; Dantas *et al*., 2008; Li *et al*., 2009; Dong *et al*., 2011;Gao et al.2012 and Hemala *et al*., 2014). 16srRNA gene phylogenetic analysis suggested that the two isolated *Chitinophaga* strains from polluted soil closely related to *Chitinophaga giangnigensis* (Figure 1).Our results are in conformity with the results of Lee et al (2009) and Yasir et al (2011) who observed the similar chemotaxonomic characteristics of some novel sp *Chitinophaga* isolated from soil. According to the present study, it can be interpreted that the isolated strains of *Chitinophaga* sp. play an important role in the successful survival and growth of plants in contaminated soils of paper mill by alleviating the metal toxicity and supplying the plant with nutrients.

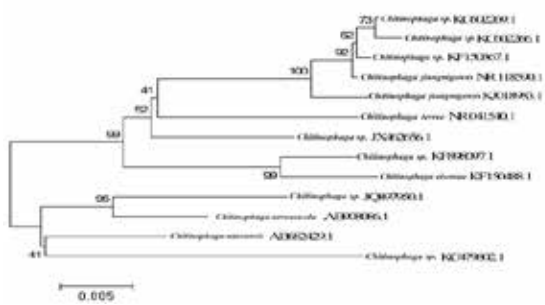

Figure 1: Neighbour-joining tree of 16SrRNA gene sequences from isolates of Chitinophaga sp (KC602266 and KC602269) with 16SrRNA of other bacteria obtained from gene bank. The Kimura two-parameter substitution model was used and the nodes are supported by 1,000 bootstrap replications. Bootstrap values above 50% and the genetic distance scale are shown (Mega 4.1 version).

CONCLUSION:

From the present study, it can be concluded that the two isolated strains of *Chitinophaga* sp are able to tolerate higher concentrations of heavy metals. On the basis of phenotypic and genotypic features, these isolated strains of *Chitinophaga* sp (KC602266 and KC602269) can be termed as heavy metal tolerant strains that might be utilized as potential bioremediation agent of paper mill polluted soil contaminated with various heavy metals.

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