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Isolation and molecular characterization of bacteria to heavy metals isolated from soil samples in Bokaro Coal Mines, India

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ABSTRACT: In recent years, environmental pollution by coal mining is a long-established human activity affecting all levels of life with various environmental impacts by generating heavy metals. The presence of heavy metals even in trace amount is toxic and detrimental to all living organisms. The coal mine area in Bokaro is one of the "Toxic Hotspot" in India. Bacteria have evolved uptake and efflux mechanisms to adapt in heavy metals contaminated environments and thus represent a potential source for bioremediation processes. In the present study, we isolated and characterized eight heavy metal resistant bacteria (NK-1 to 8) from soil sample in Bokaro coal mines, India. Isolates were selected based on high level of heavy metal resistance and its biochemical characterization. The following bacteria were identified based on 16S rRNA gene sequencing *Enterobacter ludwigii* (KM029957; NK-1), *Klebsiella pneumonia* (KM029958; NK-2), *Enterobacter ludwigii* (KM029959; NK-3), *Enterobacter ludwigii* (KM029960; NK-4), *Klebsiella oxytoca* (KM029961; NK-5), *Enterobacter cloacae* (KM029962; NK-6), *Acinetobacter gyllenbergii* (KM029963; NK-7), *Enterobacter cloacae* (KM029964; NK-8). A high degree of metal resistance associated with multiple antibiotic resistances was also detected in the selected isolate which was confirmed by the presence of plasmid. These isolates can further be used for bioremediation of heavy metals from contaminated site.

Keywords: bioremediation, coal mine, environment, heavy metals, 16S rDNA.

INTRODUCTION

The world's demand for coal, the ''black gold,'' has been on the increased since the start of the industrial revolution. The coal mining activity is characterized by the generation of large amount of by-products. One of the effects is the increase in heavy metal solubility, which results in the accumulation of these toxic elements in the environment. As a consequence of this, the environment has become acidic and rich in heavy metals. The toxic effects of heavy

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metals mainly result from the interaction of metals with proteins (enzymes) and inhibition of metabolic processes. High levels of heavy metals not only threaten human health through the food chain, but also decrease crop production and soil microbial activity (Mclaughlin et al., 1999; Giller et al., 1998; Yao et al., 2012; Nwachukwu et al., 2010). However, in order to survive in heavy-metal polluted environments, many microorganisms have developed means of resistance to toxic metal ions, they respond to the heavy metals and detoxify them by several processes; including transport across the cell membrane, biosorption to the cell walls and entrapment in extracellular capsules, precipitation, complexation and oxidation-reduction reactions (Nies and Silver, 1995; Silver, 1996; Veglio et al.,1997; Nies, 1999; Bruins et al., 2000; Spain, 2003; Yao et al., 2012; Nwachukwu et al., 2010; Oyetibo et al., 2010).

Bacteria that are resistant to heavy metals also play an important role in biogeochemical cycling of those metal ions (Issazadeh et al., 2013; Kumar et al., 2011). Thus, it is worthy to note that although some heavy metals are essential trace elements and they are toxic at high concentrations to microbes, some microbes have however adapted to tolerate the presence of metals and use them to grow, these interactions between microbes and metals have important environmental implications basically in bioremediation. Thus, the bioremediation of heavy metals using microorganisms has received a great deal of attention in recent years. Knowledge of the phenotypic, genotypic and biological characteristics of bacteria is imperative in differentiating it from its relatives or other microorganisms. As such, accurate identification of the bacteria is a fundamental component of all assessments of bacterial community. Furthermore, the conclusions pertaining to product safety or its impacts on human health or the environment are valid only if the active microorganism(s) are correctly identified. Before the invention of DNA sequencing technique, bacteria were mainly classified based on their shapes, also known as morphology, staining and biochemistry. The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods. Over the past decade the use of genes encoding 16SrDNA as molecular markers has become a modern technique for microbial ecologists offering new perspectives on the traditional phenotypic classification system (Woese, 1987). Ribosomal DNA sequence information facilitates not only a more complete understanding of microbial phylogeny, but also the identification of bacteria in environmental samples (Woese, 1987). There have been numerous studies on heavy metal resistance of bacteria isolated from various habitats (Shi et al., 2002; Ezaka et al., 2011; Rajaganapathy et al., 2011; Adu et al., 2012; Mgbemena et al., 2012; Murthy et al., 2014). However, there is no information available on heavy metal resistant bacteria from coal mine area of Bokaro, India.

Therefore, the objectives of this study is to isolate, identify and characterize heavy metals resistant bacteria from soil sample in Bokaro coal mines which might be useful in heavy metal bioremediation from the contaminated site.

MATERIALS AND METHODS

Field site, soil samples and heavy metal content measurement

The soil samples, down to 10 cm depth, were collected from Bokaro Coal Mines, India. The sampling site is situated near a dump containing heavy-metal-contaminated waste. The soil sample contained high concentrations of nickel (2,109 mg/kg), cobalt (355 mg/kg) and zinc (177mg/kg); iron (1750 mg/kg); lead (140 mg/kg) and

copper (160 mg/kg); cadmium (8 mg/kg), manganese (662 mg/kg).

Preparation of soil-borne bacterial suspension

Five grams of the soil was mixed in a sterile 250 ml flask with 90 ml of a 0.85% (w/v) salt solution and incubated at 30°C in a shaker incubator at 120 rpm for 2 h. The suspensions obtained were filtered through Whatman No.1 filter paper under sterile conditions, and these filtered bacterial suspensions were used for further work.

Isolation of bacteria

Subsamples (1.0 ml) withdrawn from the soil-borne bacterial suspension were serially diluted (in range: 10^{-1}–10^{-6}) and each dilution was plated in triplicate on Luria Bertani solid medium (HiMedia, India) containing 1.5% agar and incubated at 37°C for 48 h. The pH of the final medium was adjusted to 7.2. If appropriate, 50 mg/l of actinomycin D was added to the medium to preclude the growth of fungi. The developed colonies were counted in plates and the average number of colonies per three plates was determined. The total number of bacteria was determined as CFUs. Individual colonies of bacteria showing and having different morphological appearance on agar were selected and purified on the same media by streaking 3-4 times in the fresh media. The bacterial isolates were kept on slants at 4°C and re-cultured every 4 weeks.

Biochemical characterization of bacterial isolates Bacterial isolates were grown on Luria Bertani agar media (HiMedia India). The shape and colours of the colonies were examined under the microscope after gram staining. Isolates were made to undergo biochemical analyzed such as citrate utilization, lysine utilization, ornithine utilization, urease, phenylalanine deamination, nitrate reduction, hydrogen sulfide production, glucose, adonitol, lactose, arabinose, sorbitol utilisation using KB002 Hi-Assorted biochemical test kit (HiMedia, India) as per manufacturer's instruction.

Determination of antibiotic resistance

The antibiotic resistance was done by standard agar disc diffusion method on Luria Bertani agar using commercial discs (Himedia, Mumbai). One hundred micro liters of fresh bacterial cultures were spread on Luria Bertani agar. The following antibiotics such as neomycin (30 µg/disc), streptomycin (10 µg/disc), kanamycin (30 µg/disc), ampicillin (10 µg/disc), and tetracyclin (10 µg/disc) were placed on the plate. The plates were incubated at 37°C for 24 h. Inhibition zones in diameters were measured in mm. Strains were classified as resistant (R), Intermediate (I) and Susceptible (S) according to the criteria recommended by the national committee for clinical laboratory standards, 2001.

Minimal inhibitory concentrations of heavy metals of bacterial isolates

Minimal inhibitory concentrations (MICs) of Cd^{2+}, Pb^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} for microbial isolates were determined by the plate dilution method as described by Aleem et al. [2003]. Heavy metals Cd^{2+}, Pb^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} were used as cadmium nitrate, lead nitrate, ferrous sulphate, manganese sulphates monohydrate, copper sulphate respectively. The MICs were expressed as the lowest concentration of heavy metals that completely prevented the growth of bacteria.

Isolation of plasmid DNA

Plasmid miniprep method was used for isolation of plasmid DNA from bacteria as previously described (Dillon, 1985). The isolated plasmid DNA was analyzed by 1% agarose gel electrophoresis according to the standard procedure of Sambrook and Rissell (2001).

Transformation of plasmid DNA

To investigate the plasmid-mediated

resistance of bacterial to heavy metals under study, competent cells of *E. coli* DH5α, sensitive to heavy metals, were transformed with respective plasmid using the standard method previously described by Sambrook and Rissell (2001). One hundred micro liters of transformed *E. coli* DH5α suspensions were plated on Luria Bertani media supplemented with 3 mM cadmium nitrate, 4 mM lead nitrate, or 5 mM ferrous sulphate, 80 mM manganese sulphates monohydrate, 2 mM copper sulphate and incubated at 37°C for 24 h. Plasmid characterization of transformant and non-transformant *E. coli* DH5α were compared with a resistant strain by 1% agarose gel electrophoresis.

Molecular characterization (16S rRNA) of bacterial isolates

For 16S rRNA gene amplification, genomic DNA was extracted from bacterial isolates as previously described by Sambrook and Rissell (2001). The genomic DNA was re-suspended in 50 ml of TE Buffer (10 mM Tris, 1 mM EDTA), pH 8.0 and stored at -20°C until it was ready for use in PCR amplification.

The 16S rRNA gene of about 1.5 kb long was PCR amplified using the universal primers: forward primer (27f: 5' AGAGTTTGATCCTGGCTCAG 3') and reverse primer (1492r: 5´GGTTACCTTACGACTT3´).

Amplification was performed in a 25 µl reaction volume containing 100 ng DNA, 2.5 µl 10X PCR buffer, 40 µM $MgCl_2$, 5 µM dNTPs mix, 1.5U Taq DNA polymerase and 1 µl of 10 pmol of both forward and reverse primer and 14.7 µl of distilled water. The PCR reactions were carried out at an initial denaturation step at 94°C for 6 min, followed by 25 cycles at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min, the reaction was completed by final extension at 72°C for 8 min. The PCR products of 16S rRNA gene were then electrophoresed on 1.2% agarose gel and purified using a SureExtract PCR gel extraction kit (Genetix) according to the manufacturer's instructions.

DNA sequencing and phylogenetic analysis

The ~1.5 kb-PCR products of 16S rRNA genes were used for DNA sequencing. Sequence analysis of the DNA fragments was performed and compared against the GenBank database using the NCBI Blast program. Selected sequences of other microorganisms with greatest similarity to the 16S rRNA sequences of bacterial isolates were extracted from the nucleotide sequence databases and deposited in GenBank under accession numbers KM029957 (*Enterobacter ludwigii*; NK-1), KM029958 (*Klebsiella pneumonia;* NK-1), KM029959 (*Enterobacter ludwigii;* NK-1), KM029960 (*Enterobacter ludwigii;*NK-1), KM029961 (*Klebsiella oxytoca;* NK-1), KM029962 (*Enterobacter cloacae;* NK-1), KM029963 (*Acinetobacter gyllenbergii;* NK-1) and KM029964 (*Enterobacter cloacae;* NK-1). These 16S rRNA sequences of bacterial isolates was aligned for construction of phylogenetic tree using clustal W by distance matrix analysis and the neighbor joining method. Phylogenetic trees were displayed using TREEVIEW.

RESULTS

Isolation and identification of heavy metal resistance bacteria

In the present study, we identify and characterize heavy metal resistant bacteria isolated from soil samples in the coal mines. Three hundred colonies were screened from an initial level of heavy metal supplemented LB medium. Eight isolates designated as NK-1 to NK-8, were selected based on high degree of heavy metals resistances and were used for further studies (Table 1).

MIC of heavy metal

Isolates NK-1, NK-2, NK-3 and NK-5 showed very high degree of resistance to cadmium and Lead whereas NK-4, NK-6,

Table 1. Molecular identification of the isolates based on partial 16S rDNA analysis

Strain	Bacterial strain showing maximum homology	Identity (%)	GenBank accession No.
NK-1	*Enterobacter ludwigii*	98	KM029957
NK-2	*Klebsiella pneumonia*	94	KM029958
NK-3	*Enterobacter ludwigii*	99	KM029959
NK-4	*Enterobacter ludwigii*	99	KM029960
NK-5	*Klebsiella oxytoca*	99	KM029961
NK-6	*Enterobacter cloacae*	97	KM029962
NK-7	*Acinetobacter gyllenbergii*	93	KM029963
NK-8	*Enterobacter cloacae*	99	KM029964

NK-7 and NK-8 showed very high degree of resistance to Fe^{2+}, Mn^{2+} and Cu^{2+}. MIC values vary in concentration from 2-80 mM. Among the heavy metals, Fe^{2+} and Mn^{2+} were less toxic, whereas cadmium, lead, and copper were highly toxic to all isolates. The MIC values for iron and manganese metals were 5 and 80 mM respectively while the MIC values for Cadmium, lead and copper were 3, 4 and 2 mM respectively (Table 2).

Table 2. Minimal inhibitory concentrations (MICs) of heavy metal of the bacterial isolates

MICs of heavy metals	CFU of bacterial
Cd^{+3}	
1	115 (12)*
2	277(12)
3	6 (3)
4	0
Fe^{+2}	
1	92 (9)
2	65 (9)
3	48 (6)
4	38 (3)
5	6 (1)
6	0
Pb^{+2}	
1	91 (9)
2	56 (9)
3	24 (3)
4	8 (1)
5	0
MN^{+2}	
20	542 (6)
40	126 (6)
60	13 (6)
80	7 (2)
90	0
Cu^{+2}	
1	18 (3)
2	7 (1)
3	0

Values in parentheses represent the type of resistant isolates.

Antibiotic resistance assay

Isolated strain showed varying degrees of susceptibilities to antibiotics. Isolated strain appeared to be susceptible, being inhibited by all antibiotics. Strains were sensitive to Neomycin, Streptomycin, Tetracyclin and Kanamycin, while they are intermediately sensitive to ampicillin. Depending on the antibiotics, the isolates showed different degree of tolerance to antibiotics (Table 3).

Biochemical characterization

After performing the gram staining, all the bacterial isolates were found to be gram negative. Several Biochemical tests were carried out to characterize strains.

Isolates NK-1-3 were positive for citrate utilization, as well as lysine and ornithinedecarboxylation.

Table 3. Antibiotic sensitivity profile of heavy metal resistant bacterial isolates

Antibiotics	Diameter of inhibition zone (cm)							
	Nk-1	**Nk-2**	**Nk-3**	**Nk-4**	**Nk-5**	**Nk-6**	**Nk-7**	**Nk-8**
Neomycin (30mcg/disc)	2	1.8	2.2	1.8	2	1.7	1.9	2.3
Streptomycin (10mcg/disc)	2.3	1.7	1.5	1.8	2	1.9	2.2	1.8
Kanamycin (30mcg/disc)	1.7	2	1.7	2.1	1.8	1.6	2	2.2
Ampicillin (10mcg/disc)	1.2	1	1.1	1.5	1.4	1.4	1.5	1.4
Tetracyclin (10mcg/disc)	1.5	1.5	1.4	2	1.6	1.7	1.8	1.7

mcg stands for microgram and mm stands for centimeter; 1.3 cm or less zone of inhibition represent resistant isolates; 1.4 to 1.6 cm zone of inhibition represent Intermediate isolates; >1.7 cm zone of inhibition represent susceptible isolates.

Table 4 shows the utilization of several substrates by bacterial isolates including nitrate reduction ability, and the utilization of lactose, arabinose and sorbitol. NK-6, NK-7 and NK-8 showed positive reaction for utilization of citrate, lysine, ornithine, nitrate reduction, lactose, arabinose and sorbitol utilization and variability in utilisation of glucose (Table 4).

Detection of plasmid in heavy metal resistance bacteria and their role in metal resistance

All the different isolates tested for the presence of plasmids, showed three distinct band of open circular, linear and supercoiled plasmid on 1% agarose gel (Fig. 1). Through the transformed competent cells, the ability of the isolates to be resistant to the heavy metals was confirmed to be mediated by plasmid as the *E. coli* DH5α cells transformed with the plasmid grow on

LB medium supplemented with 3 mM Cd, 4 mM Pb, 5 mM Fe, 2 mM Cu and 80 mM Mn, while the non-transformed strain could not grow on these heavy metals supplemented media. The presence of plasmid in the transformed strain was confirmed by plasmid preparation and agarose gel electrophoresis, and indicated that the transformed strain acquires plasmid of the same size as in the isolated resistant strains.

Bacterial identification by 16S rDNA analysis

PCR amplification of the 16S rDNA gene produced fragments of approximately 1500 base pairs in size (Fig. 2). The partially amplified sequence of 16S rRNA gene from isolates (NK-1 to 8) were aligned and the closest match was detected using BLAST. The nucleotide sequences coding for the 16S rRNA gene after BLAST query revealed that this gene is 98% homologous to *Enterobacter ludwigii* (KM029957; NK-1), 94% homologous to *Klebsiella pneumonia*

Table 4. Biochemical characteristics of heavy metal resistant bacterial isolates

Tests	Isolates	NK-1	NK-2	NK-3	NK-4	NK-5	NK-6	NK-7	NK-8
Citrate utilization		+	+	+	+	+	+	+	+
Lysine utilization		+	+	+	+	+	+	+	+
Ornithine utilization		+	+	+	+	+	+	+	+
Urease		+	+	+	-	-	-	-	-
Phenylalanine deamination		-	-	-	-	-	-	-	-
Nitrate reduction		+	+	+	+	+	+	+	+
Hydrogen sulfide production		-	-	-	-	-	-	-	-
Glucose utilization		-	-	-	-	-	-	±	±
Adonitol utilization		±	±	±	-	-	-	-	-
Lactose utilization		±	±	±	+	+	+	+	+
Arabinose utilization		+	+	+	+	+	+	+	+
Sorbitol utilization		+	+	+	+	+	+	+	+

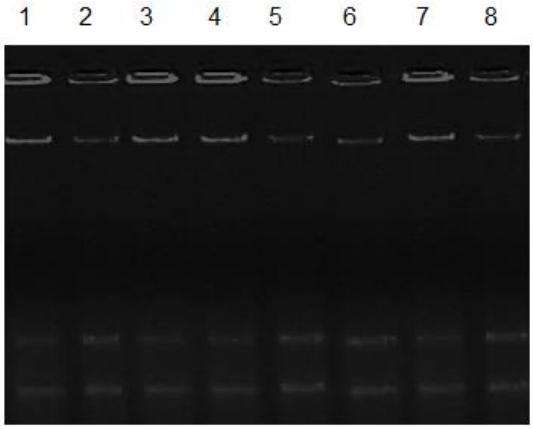

Fig. 1. Isolation of Plasmid DNA from heavy metal resistant isolates. Lane 1: NK-1, Lane 2: NK-2, Lane 3: NK-3, Lane 4: NK-4, Lane 5: NK-5, Lane 6: NK-6, Lane 7: NK-7, Lane 8: NK-8

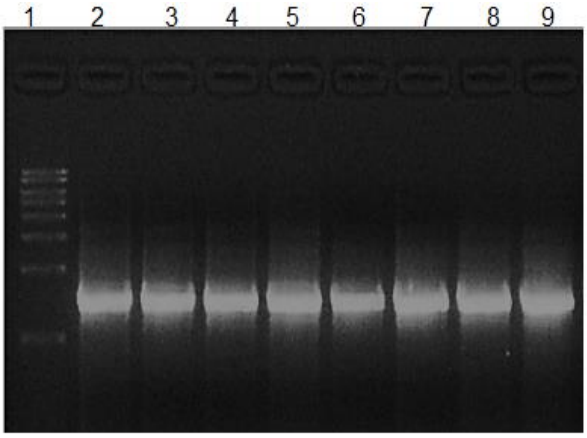

Fig. 2. PCR amplicons of bacterial isolates 16S rDNA genes. Lane 1: 1 kb ladder, Lane 2: NK-1, Lane 3: NK-2, Lane 4: NK-3, Lane 5: NK-4, Lane 6: NK-5, Lane 7: NK-6, Lane 8: NK-7, Lane 9: NK-8

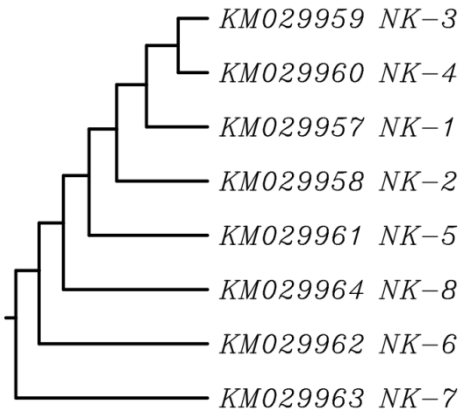

Fig. 3. Phylogenetic tree based on 16S rDNA sequences

(KM029958; NK-2), 99% homologous to *Enterobacter ludwigii* (KM029959; NK-3), 99% homologous to *Enterobacter ludwigii* (KM029960; NK-4), 99% homologous to *Klebsiella oxytoca* (KM029961; NK-5), 97% homologous to *Enterobacter cloacae* (KM029962; NK-6), 93% homologous to *Acinetobacter gyllenbergii* (KM029963; NK-7), 99% homologous to *Enterobacter cloacae* (KM029964; NK-8). A phylogenetic tree was constructed by aligning 16S rRNA sequences of bacterial isolates (Fig. 3).

DISCUSSION

In recent years, heavy metal pollution has become one of the most serious environmental problems. Pollution of the environment with toxic heavy metals is spreading throughout the world along with industrial progress. Presence of heavy metals even in trace amounts is toxic and detrimental to all living organisms (Spain, 2003, Islam et al., 2007, Iwegbue et al., 2010). Moreover, it cannot be degraded into simpler harmless compounds like other pollutants including, diverse range of organics, polyaromatic hydrocarbons (PAHs), organo-chlorines, dyes etc (Pandit et al., 2013). The coal mines in Bokaro area is a "Toxic Hot spot" in the state. In the present study, we isolated and characterized eight heavy metal resistant bacteria from soil sample at Bokaro coal mines, India. Isolates were selected based on high level of heavy metal resistance, their morphological and biochemical characterization was also done. Identification based on 16S rRNA gene sequencing identified the following bacteria; *Enterobacter ludwigii* (KM029957; NK-1), *Klebsiella pneumonia* (KM029958; NK-2), *Enterobacter ludwigii* (KM029959; NK-3), *Enterobacter ludwigii* (KM029960; NK-4), *Klebsiella oxytoca* (KM029961; NK-5), *Enterobacter cloacae* (KM029962; NK-6), *Acinetobacter gyllenbergii* (KM029963; NK-7),

Enterobacter cloacae (KM029964; NK-8). A phylogenetic tree was constructed by aligning 16S rRNA sequences of bacterial isolates (Fig. 3). Our results are similar to the findings of Pandit et al. (2013). Isolates showed high degree of resistance to heavy metals under investigation. A high degree of metal resistance associated with multiple antibiotic resistances was also detected in the selected isolate (Table 3). In most of the studies, metal resistance is linked with antibiotic resistance (Sharma et al., 2000; Verma et al., 2001; Oyetibo et al., 2010). Similarly Filali et al. (2000) studied waste water bacterial isolates which were resistant to heavy metals and antibiotics. The exposure to heavy metals results in the selection of bacterial strain which were also able to resist antibiotics. This happens because genes encoding heavy metals are located together with antibiotic resistance genes. Under conditions of metal stress, metal and antibiotic resistance in microorganisms possibly help them to adapt faster by the spread of resistant factors than by mutation and natural selection (Silver and Misra, 1988). The genetic determinants of resistance are frequently located on plasmids (Cervantes et al., 1991). In the present study, it was observed that resistance found in isolates was due to plasmid. Plasmid was present in all the isolates. Bacterial plasmids have genes that confer highly specific resistances to toxic heavy metals. For each toxic cation and anion, generally a different resistance system exists, and these systems may be linked together on multiple resistance plasmids (Silver et al., 1989).

CONCLUSION

The overall study shows that isolates have high capacity to tolerate metals under investigation hence, these isolates can be used in bioremediation of contaminated soil of coal mines having toxic heavy metals. The antibiotic sensitivity profile of these isolates can provide serious insights to explore the molecular mechanisms of resistance and correlation between metal and antibiotic resistance.

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