Research Article

The P-type ATPase Mediates Copper Tolerance in *Klebsiella pneumoniae* IFCu4 Strain Isolated from South Gujarat Industrial Effluents

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ABSTRACT

South Gujarat is one of the heavily industrial based regions in India rendering a man-polluted environment. Industrial effluent samples were used to isolate copper tolerant bacteria. The isolate IFCu4 tolerated copper ($CuSO_4$) at 28 mM in minimal media. The isolated strain was identified as *Klebsiella pneumoniae* through 16S rRNA gene sequencing and the sequence was submitted to NCBI database (Accession number: KF496212). The strain IFCu4 (*Klebsiella pneumoniae*) also possesses the integral inner membrane copper-transporting P-type ATPase, which was confirmed by polymerase chain reaction with specific primers for *copA* gene. The presence of *copA* gene encoding copper-transporting P-type ATPase contributes in removal of excess Cu (I) from the cytoplasm to protect microorganisms from copper–mediated toxicity at high concentrations; hence it is proposed that the isolated strain has potential role in bioremediation.

Keywords: Copper, *Klebsiella pneumoniae*, copA

1. INTRODUCTION

Industrialisation processes throughout the world increases different types of inorganic and organic pollutants, toxic heavy metals like cadmium, nickel, lead, chromium, mercury, manganese, zinc, iron and copper. These pollutants create hazardous environmental pollution which is toxic to living organisms [1, 2]. Copper is the 5th highest toxic metals [3]. Copper is included on the US Environmental Agency (EPA) list of priority pollutants [4]. Apart from its toxic nature, copper is also an essential micronutrients for living organisms at very low concentration. The high concentration of copper can generate reactive oxygen species (ROS) leading to oxidative damage to biological systems, including peroxidation of lipids or other macromolecules [5] displacement of other metal cofactors from their natural ligands in signalling proteins [6] oxidation of proteins and cleavage of DNA and RNA molecules [7] resulting in general cellular damage and metabolic perturbation in various biochemical pathways. Moreover, ROS are thought to play a major role in cancer development and in aging process [8].

Copper resistance genes in bacteria are often

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present in plasmids or on chromosome and organized in an operon [9-11]. Active efflux of the metal from the cytoplasm to the periplasmic space is carried out by ATPases located in the internal membrane of the bacteria is the known pathways for Copper resistance in Gram-negative bacteria [12]. The *copA* encodes a P-type ATPase [13] that transports cytoplasmic copper across biological membrane to periplasmic space and cueO is a multi-copper oxidase that oxidise Cu(I) to the less toxic chemical form of Cu (II) [14-16]. A *copA* gene involved in Copper homeostasis has been described in *Escherichia coli, Pseudomonas syringae, Pseudomonas fluorescens, Sulfitobacter* sp. NAS-14.1 and *Xanthomonas campestris* [9].

The present work reported an enrichment technique to isolate copper tolerant bacteria from industrial effluent and explored its tolerance mechanism by using gene specific PCR amplification of *copA* gene, detected in *K. pneumoniae* which is involved in the transports of copper from cytoplasm to periplasm.

2. MATERIALS AND METHODS

2.1. Isolation of Copper-tolerant bacteria

Industrial effluents were collected in screw capped sterile bottles from different sites located at Sachin, Surat, Gujarat, India. The stock solution of $CuSO_4$ was buffered at pH 7 using 4 mole equivalents of glycine to avoid precipitation of Cu (II) salts [17]. Copper-tolerant bacteria were isolated by enrichment technique, 1 ml of sample was inoculated into Minimal medium (Dextrose 1g, Dipotassium phosphate 7g, Monopotassium phosphate 2g, Sodium citrate 0.50g, Magnesium sulphate 0.1g, Ammonium sulphate 1g, 1000 ml Distilled water with 1 mM of copper sulphate ($CuSO_4$) and incubated at 30°C on a rotary shaker at 150 rpm for 48 h. The grown culture was sub-cultured with increasing concentrations of copper from 1 to 30 mM [18]. Then grown culture was harvested and transferred to fresh copper sulphate amended medium. The culture was sequentially purified by streaking on Copper sulphate supplemented Minimal agar plates, isolated colonies which could tolerate maximum amount of Copper were purified by repeated sub culturing.

2.2. Identification of bacteria

DNA was isolated from cells grown on Minimal medium for 24 h by CTAB-NaCl method [19]**.** PCR amplification of 16S rRNA gene was done using universal primer pair 27f and 1492r in a 50 µL reaction with an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1.30 min, 72°C for 1 min, with a final extension of 72°C for 10 min. The purified 16S rRNA gene fragment were used as templates for DNA sequencing with an ABI Prism 3130 automatic sequencer (Applied Biosystems, U.S.A) using forward primers. The nucleotide sequence was deposited in the GenBank, NCBI sequence database. Phylogenetic tree was constructed by the neighbour-joining (NJ) method of the MEGA 5.2 software programme.

2.3. Minimum inhibitory concentration (MIC) of other heavy metals for bacterial strains

The MIC of other toxic metals such as Na_2AsO_3, $K_2Cr_2O_7$ and $NiCl_2$ were also estimated in bacterial isolate which was able to grow in presence of $CuSO_4$ (28 mM) [20]. The isolated bacterium was grown in Minimal medium. An aliquot of overnight grown culture were inoculated in Minimal broth, supplemented with different concentration of Na_2AsO_3, $K_2Cr_2O_7$ and $NiCl_2$ and inoculated flasks were incubated at 30° C and checked for growth at 600 nm after 72 h.

2.4. Characterization of Copper tolerance bacterial stain IFCu4

The growth curve of *K. pneumoniae* was plotted by growing the cells in Minimal medium with 0 mM, 5 mM, 10 mM and 15 mM $CuSO_4$ with continuous shaking at 150 rpm in the orbital shaker at 30^o C. 2 mL culture broth was collected at different time intervals. Growth was monitored by measuring optical density (OD) of the cultures at 600 nm using a spectrophotometer (Shimadzu UV-1800, Japan).

Biochemical tests such as Gram staining, catalase test, oxidase test, indole, methyl red, Voges–Proskauer, nitrate reduction, H_2S production, hydrolysis of casein, gelatin, and utilization of various carbon compounds, were performed as previously described [21-23].

2.5. Antibiotic sensitivity

The bacterial strain showed copper tolerance was inoculated in the Mueller Hinton broth and incubated at 30° C for overnight. Overnight grown culture was reinoculated in fresh broth till the culture reach to 0.5 to 0.6 O.D at 600 nm and was spread on Mueller Hinton agar plates. The plates were allowed to dry for 10 minutes and then antibiotic discs were placed on the plates. The plates were incubated at 30° C for 16 h and the inhibition zone was measured around the discs. The identification of strain as susceptible or resistant was done in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines.

2.6. Gene-specific PCR amplification of copper tolerance gene

The molecular characterization on Copper tolerance ability of bacterial isolate was performed by colony PCR using *copA* gene specific primer [24].

3. RESULTS AND DISCUSSION

3.1. Isolation of Copper tolerance bacteria

In this study, we have attempted to isolate and characterize copper tolerant bacteria and explored the mechanism of tolerance. During the selection strategy one of the isolate showed better tolerance to copper at very high concentration. Further, we observed that in minimal broth, 30 mM $CuSO_4$ was sufficient to inhibit the growth of selected bacterial strain while on minimal agar media, 20mM $CuSO_4$ was sufficient to inhibit the growth of that bacterial strain. Cross tolerance were reported in the selected bacterial strain for heavy metals toxicity [25]. Similarly we observed in *K. pneumoniae* IFCu4 not only tolerate high $CuSO_4$ at a concentration of 28mM but also showed tolerance against Na_2AsO_3, $K_2Cr_2O_7$ and $NiCl_2$ at a concentration of 18.75mM, 0.5mM and 3.08mM respectively.

3.2. Characterization of Copper tolerance bacterial stain IFCu4

Growth curves were plotted for the bacterial strain at different concentration (0 mM, 5 mM, 10 mM, and 15 mM) of $CuSO_4$ to estimate the time when they reach log phase (Figure 1). In the presence of 5 mM of copper sulphate, the organism growth was retarded in culture broth while in 10 mM and 15 mM of copper sulphate concentration the lag phase was extended up to 3 h as compared with the control cultures in which no $CuSO_4$ were added. Log phase is ideal time to perform any experiments of heavy metal (copper sulphate) tolerance. Log phase cultures were utilised to calculate MIC of isolated bacterial strain. The relationship between generation time and increasing metal concentration was positive. The differences in the lag phase can be an indication of the induction of metal-specific mechanisms eliciting cellular protection before growth resumes [26].

Strain IFCu4 was found to be Gram-negative, facultative anaerobic and rod shaped bacterium. The isolate was positive for utilization of D-glucose, D-mannitol, lactose, sucrose, maltose, and xylose with gas production. The isolate was positive for Nitrate, Voges–Proskauer and Catalase and negative for Methyl red, Indole, Citrate, Phenyl alanine, H_2S production, Oxidase, Urea, Starch Hydrolysis, Lipid Hydrolysis, Gelatin and Blood agar plate.

The isolate showed resistant to Ampicilin [10 µg], Cefalexin [30µg], Sulphatrial [300µg], Clindamycin [2µg] and Cloxacilin [5µg] and sensitive to Tetracycline [30µg], Chloramphenicol [30µg], Gentamycin [10µg], Co-Trimoxozole [25µg], Streptomycin [10µg], Penicillin G [1µg], Erythromycin [15µg], Nalidixic acid [30µg], Ceftriaxone [30µg], Cefotaxime [30µg] and Ciprofloxacin [5µg] (Table 1).

Table 1: Antibiotic susceptibility pattern of *K. pneumoniae* strain IFCu4.

Antibiotics	Result	Antibiotics	Result
Nalidixic acid[30µg]	S	Penicillin G[1 µg]	S
Ampicilin[10 µg]	R	Tetracycline[30 µg]	S
Chloramphenicol[30 µg]	S	Ciprofloxacin[5 µg]	S
Erythromycin[15 µg]	S	Sulphatrial[300 µg]	R
Streptomycin[10 µg]	S	Clindamycin[2 µg]	R
Gentamycin[10 µg]	S	Ceftriaxone[30 µg]	S
Co-Trimoxozole[25 µg]	S	Cloxacilin[5 µg]	R
Cefalexin[30 µg]	R	Cefotaxime[30 µg]	S

Key: S = Sensitive, R = Resistance

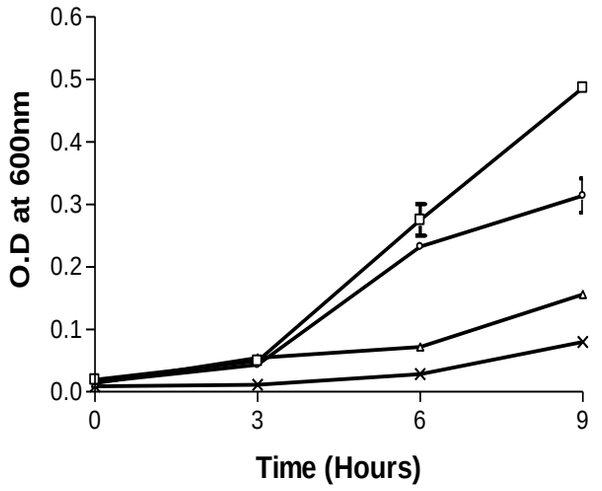

Figure 1: Effect of copper concentration on the growth rate of *K. pneumoniae* strain IFCu4. Growth rates were determined in Minimal media supplemented with 0mM (□), 5mM (○), 10mM (Δ), and 15mM (×) copper sulphate. The values are expressed as mean ± SEM.

3.3. Identification of Copper tolerance bacterial strain IFCu4

In order to identify the bacterial species IFCu4, it was subjected to 16S rRNA gene sequence analysis. The 16S rRNA gene sequence of strain IFCu4 was submitted to GenBank (Accession number: KF496212). The information obtained by the BLAST program indicated that it shared maximum similarity (98%) with *K. pneumoniae* in NCBI database. To determine their phylogenetic position, the 16S rRNA gene sequence was analysed and phylogenetic tree was constructed using neighbour-joining (NJ) method of MEGA 5.2 software (Figure 2).

3.4. PCR Amplification of a copper tolerance gene

The copper tolerance gene *copA* in *K. pneumoniae* was amplified by colony PCR using specific primers which yielded 1.3 kb fragment (Figure 3). Thus PCR amplification confirmed that the *copA* gene present in the *K. pneumoniae* IFCu4 and encode P-type ATPase gene, which constitutes a group of periplasmic protective enzymes [27]. Previous studies showed that *E. coli* and *Salmonella* strains lacking *copA* gene are susceptible to copper toxicity [32, 33]. This reports supported our result that *copA* genes involves in tolerance of high concentration of copper.

Copper homeostasis in bacteria is assurance principally by the action of active efflux systems that remove the copper ion from the cell [14, 28]. *K. pneumoniae* strain IFCu4 possess, the P-type ATPase mediated copper tolerance that [29, 30] might involved in transport of copper ions from cytoplasm to periplasm [31].

4. CONCLUSION

K. pneumoniae strain IFCu4 able to tolerate copper due to presence of *copA* gene and could be a good candidate for bioremediation of copper-contaminated industrial wastes.

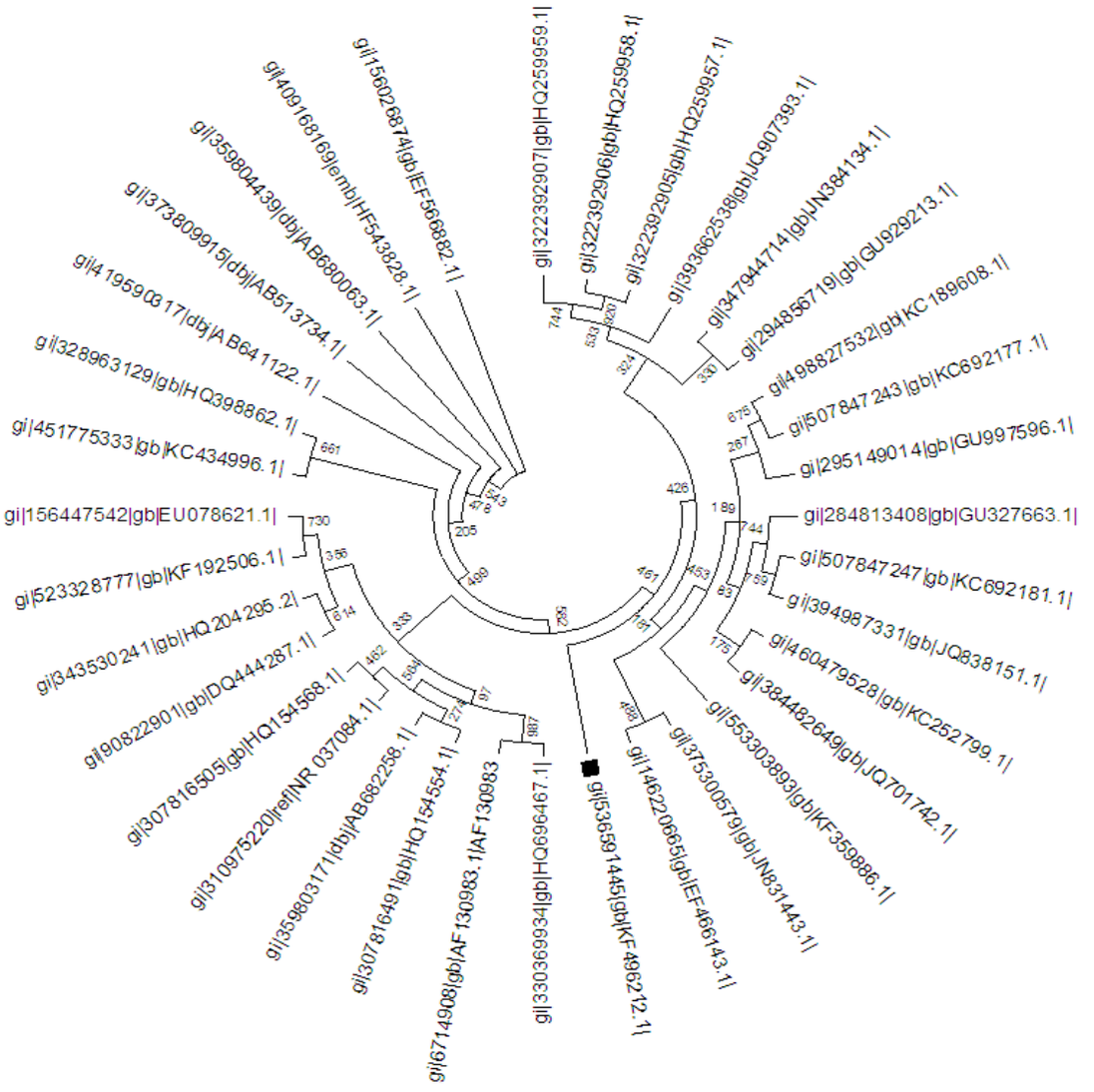

Figure 2: Phylogenetic tree of the *Klebsiella pneumoniae* strain IFCu4

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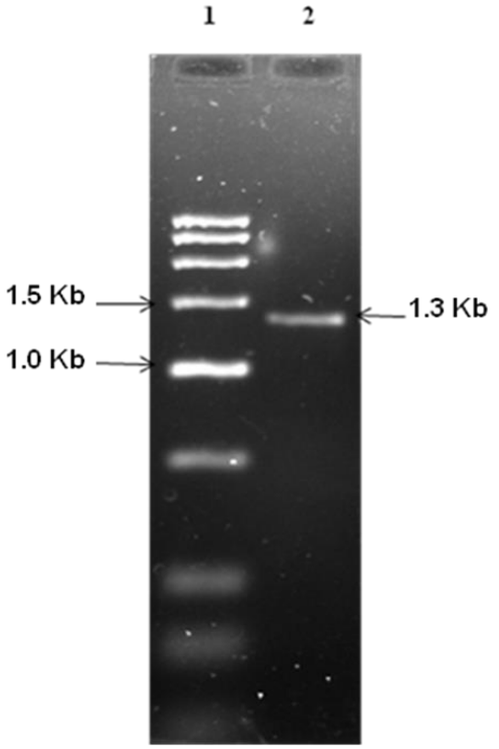

Figure 3: PCR amplification of a *copA* gene fragment: Lane 1 DNA ladder, Lane 2 *copA* gene was detected in *K. pneumoniae* strain IFCu4.

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