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RESEARCH ARTICLE

Identification of the pco operon in *Enterobacter* species isolated from contaminated soil

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Abstract

Genetic determinants of heavy metal resistance in bacteria can be localized both on chromosomal and on extrachromosomal genetic elements. Interactions between bacteria and heavy metal ions are of great interest both as a fundamental process and potential bioremedial technology. In this study copper resistance was identified in *Enterobacter* sp. isolated from oil and petrol contaminated site. The copper resistance in Enterobacter isolate designated as CMG457 was shown to be plasmid-encoded. A complete Pco operon (*pcoABCDRSE*) was identified by PCR based methods.

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Introduction

Copper is an essential micro-nutrient required by a variety of enzymes and redox reactions by bacteria. However, at higher concentrations, copper can become toxic to the cells, affecting intracellular homeostasis. Contamination of the environment with toxic levels of copper is now widespread because of mining, industrial and agricultural activities (Albright et al., 1972; Cooksey et al., 1990). This has led to the development or acquisition of copper-resistance mechanisms. Plasmid-mediated copper resistance has been reported in E. coli (Tetaz et al., 1983) Citrobacter freundii (Williams et al., 1993) and *Pseudomonas syringae* (Bender et al., 1987). The copper-resistance operons, pco in *E. coli* plasmid pRJ1004 (Brown et al., 1995) and cop in *P. syringae* plasmid pT23D (Bender et al.,1986; Mellano et al., 1988; Cooksey et al.,1994) have been characterized and sequenced. The two operons comprise of four structural genes *pcoABCD* and *copABCD* and two regulatory trans-acting genes *pcoRS* and *copRS* respectively. The pco operon also has an additional gene, *pcoE*, for which no homologue has yet been described in the cop operon. Although there is a considerable sequence homology (30-76%) between the deduced protein products of the two operons but they appears to be some differences in their respective mechanisms of resistance. In *E. coli* the pco operon enhances copper efflux (Brown et al., 1995) while in *Pseudomonas* sp. the cop operon appears to enhance copper accumulation (Cooksey et al., 1992; Cooksey, 1993). This demonstrates that numbers of copper-responsive regulators are encoded in the chromosome of different bacterial species (Munson et al., 2000; Nies et al., 1998). It has also been reported that heavy metal resistance in an organism can be due to the multiple layers of resistance systems with unique functions (Nies., 2003). Some of these systems are widespread and serve in the basic defense of the cell against superfluous heavy metals, but some are highly specialized and occur only in a few bacteria. In *Xanthomonas* sp. a chromosomally-encoded resistance mechanism similar to pco and cop has been described (Garde et al., 1991; Lee et al., 1994; Behlau et al., 2011). Copper resistance genes have also been cloned from *Xanthomonas vesicatoria* (Basim et al., 2005), *Xanthomonas arboricola* pv. *juglandis* (Lee et al., 1994) and *Xanthomonas axonopodis* pv. *vesicatoria* (Voloudakis et al., 2005). Plasmid-borne genes for copper resistance in *X. vesicatoria* have similarities to the cop operon from *P. syringae* (Voloudakis et al., 1993). The present study describes the identification of the complete pco operon on a plasmid in an environmental isolate.

Material and Methods

Selection of Bacterial strain

Several bacterial strains were checked for copper resistance and on the basis of resistance against copper salt only *Enterobacter* sp. CMG457 was selected for this study.

Assessment of metal and antibiotic resistances

In order to characterize the bacterial strain various heavy metal such as copper, cadmium, chromium, nickel, cobalt and lead and antibiotics such as ampiciline (Amp), erythromycin (Em), chloramphenicol (Cm) and streptomycin (Sm) were used. 1M stock solution of each heavy metal salts were prepared. The selective plates of tris minimal media supplemented with variable concentrations of metal salts such as 1mM to 6mM, the MTCs (maximum tolerable concentration). MTCs were determined by streaking culture on selective plates and results were observed after 24-48hrs of incubation at 37^{o}C. The antibiotic resistance was determined by preparing stock solutions of each antibiotic such as Amp (50mg/ml), Em (5mg/ml), Cm (34mg/ml) and Sm (50mg/ml) were prepared and variable working concentrations (5-100µg/ml) were used in Luria agar medium. The antibiotic selective plates were streaked and growth was observed after 24-48hrs of incubation at 37^{o}C.

Extraction of Plasmid and Genomic DNA

Plasmid DNA was extracted by a modified Birnboim and Dolly method (Birnboim et al., 1979) and by Gene JET Plasmid mini prep Kit (Fermentas Life sciences). The genomic DNA was extracted by phenol chloroform method (Sambrook et al., 1989).

Curing of plasmid DNA

Plasmid DNA was cured by using ethidium bromide (EtBr) as a mutagenic agent. Variable concentrations of EtBr ranging from 25µg/ml - 800µg/ml were supplemented in nutrient broth to estimate its maximum tolerable concentration (MTC). The culture was spread from MTC broth on nutrient agar plates and then replica plated on selective (1, 2, 4mM of $CuSO_4$) plates and control plates without copper salt. Growth was observed after 24-48 hrs of incubations.

Transformation of plasmid DNA

Transformation of plasmid DNA was performed by heat-shock method as described by Sambrook (Sambrook et al., 1989).

Designing of Primers

Oligonucleotide primers were designed to investigate the *pcoABCDRSE* genes by using primer designing program Primer 3 Output (see table 1).

PCR amplifications

PCR amplification was performed Hybaid Omn-E thermal cycler with 1µl of plasmid DNA template (in case of genomic DNA template, 0.1µl was used because of concentration) 20pmol of each primer pairs mentioned in the table 1, 200µM dNTPs, 3mM $MgCl_2$, 16mM $(NH_4)_2SO_4$, 67mM Tris –HCl (pH 8.8), 0.01% Tween-20 and 0.8U of BioTaq DNA polymerase (Bioline, London, UK). The recombinant plasmid *p*MU2385pco, which contain the entire pco operon (a 9.0kb *Hind*III-*Bgl*II fragment) cloned from plasmid pPA173 (Brown et al., 1995) was used as a positive control. Amplification condition were 94°C for 5min, 56°C for 30 s and 72°C for 3 mins for one cycle, followed by 30 cycles of 94°C for 5 min, 54°C for 30 s and 72°C for 2 min, and a final extension for 5 min at 72°C. To determine the intragenic regions of Pco operon, primer pairs of pcoB1and B2, C1 and C2, D1 and D2, E1 and E2, were used. Whereas for intergenic amplifications of primer combinations of adjacent genes were used (see figure1B). The amplified PCR product of *pcoA* gene was sequenced. The CMG457 PCR product was purified using a QIAquick PCR purification kit (QIAGEN Ltd, Boundary Court, Crawley, West Sussex, United Kingdom) and cycle sequenced according to the manufacturer's instructions using the DYEnamic ET terminator cycle-sequencing premix kit (Amersham, Little Chalfont, United Kingdom). The ABI 373 DNA sequencing system (commercially) was used to sequence both the strands using the copA forward (CopA1) and reverse (CopA2) primers.

Result and Discussion

Characterization of CMG457

Maximum tolerable concentration (MTC) of heavy metal salts was determined in order to select the strains which have multiple metal resistance and also showed highest resistance against selected heavy metals i.e., copper. The isolate CMG457 selected from the stock culture; previously it was recovered from a soil sample contaminated with oil and petrol from a site close to a petrol pump in the city of Karachi, Pakistan. The organism was identified as *Enterobacter* sp. by 16s rDNA sequence analysis. *Enterobacter* strain CMG457 was resistant to copper sulphate at 4mM in tris minimal medium (Birnboim et al., 1979) and 6mM in Luria broth. It also showed resistance against chromate, cobalt and lead salts upto 0.5 mM. In case of antibiotic resistance it showed resistance against erythromycin and ampicilline at 100 and 25 µg/ml respectively.

The location of copper resistance genes were identified by isolation of plasmid DNA, curing of plasmid and transformation experiments. The two plasmids were observed in CMG457 and designated as *p*1CMG457 and *p*2CMG457 (figure 1). The cured derivatives of CMG457 was obtained they were designated as CMG457a and CMG457b (figure 2). Both of the plasmids were cured and the cured derivative was unable to grow at higher concentration of copper sulphate i.e., at 6mM $CuSO_4$. Plasmid DNA of CMG457 was successfully transformed into XL1-Blue recipient *E. coli* strain showed copper resistance in host cells (figure 2). The transformants was designated as CMG457T showed resistance upto 4mM $CuSO_4$. It confirms the location of copper resistance genes that is on plasmid DNA (table 2).

Plasmid *p*CMG457 also appeared to carry resistance to erythromycin, since this resistance marker was also lost following plasmid curing. Furthermore, the yield of plasmid DNA extracted from CMG457 cells cultured in the presence of either $CuSO_4$ (3mM) or erythromycin (10µg/ml) was much greater than from cells cultured in the absence of selective pressure.

Screening of copper resistance genes by PCR Amplifications

In order to investigate the mechanism of copper resistance in strain CMG457, a pair of oligonucleotide primers, CopA1 and CopA2 (Table 1), were designed from the aligned *copA* and *pcoA* gene sequences. The recombinant plasmid, *p*MU2385pco, which contains the entire pco operon used as a positive control.

Following amplification a 1.3 kb PCR product was obtained from the control plasmid template, *p*MU2385pco, and from plasmid DNA extracted from isolate CMG457 and *E. coli* XL1Blue (pCMG457). A total of 1246 bases were determined which showed 100% homology with the *pcoA* gene of the *E. coli* plasmid pRJ1004 (GenBank Accession No. X83541). In order to determine whether the full pco operon was present in strain CMG457, primer pairs were used for amplifying intragenic regions of *pcoB*, *pcoC*, *pcoD*, *pcoR*, *pcoS* and *pcoE* (Table 1). PCR conditions were as before. Each primer pair generated a PCR product of the expected sizes, 1331bp, 799bp, 334bp, 874bp, 622bp, 1200bp, and 374bp respectively. Furthermore, PCR amplification with primer combinations permitting intergenic amplification of potentially adjacent genes are *AB*, *BC*, *CD*, *DR*, *RS* there sizes are given in figure 3. It confirmed that the order of genes in the pco operon present in *Enterobacter* CMG457 plasmid *p*1CMG457 was identical to the operon previously described in *E. coli* plasmid pRJ1004 (Brown et al., 1995) i.e., *ABCDRSE*. It was further confirmed by the transformants of CMG457 that showed the presence of complete Pco operon.

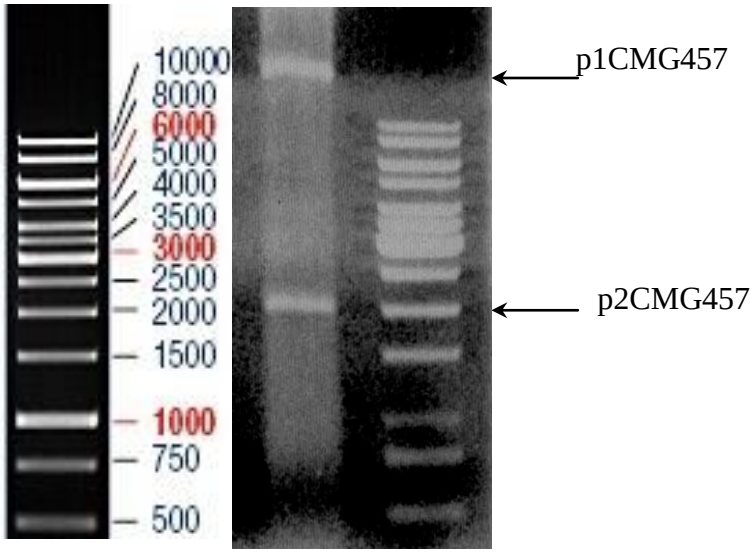

Figure 1. Isolation of Plsmid DNA of CMG457. Lane 1, CMG457, and Lane 2, 10 kb ladder (MBI), arrow showing position of two plasmid DNA of CMG457. Molecular weights of ladder on left side.

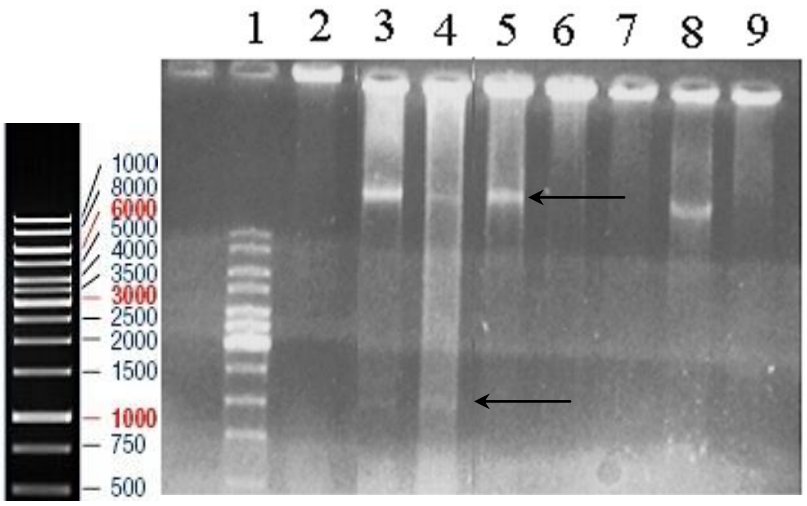

Figure 2. Extraction of plasmid DNA from CMG457. Lane1: 1kb laddar; lane 3, 4,and 5: CMG457; lane6: cured derivative (CMG457a); Lane7: cured derivative (CMG457b); Lane8: CMG457T (transformant); lane 9: XL1Blue (recipient strain).

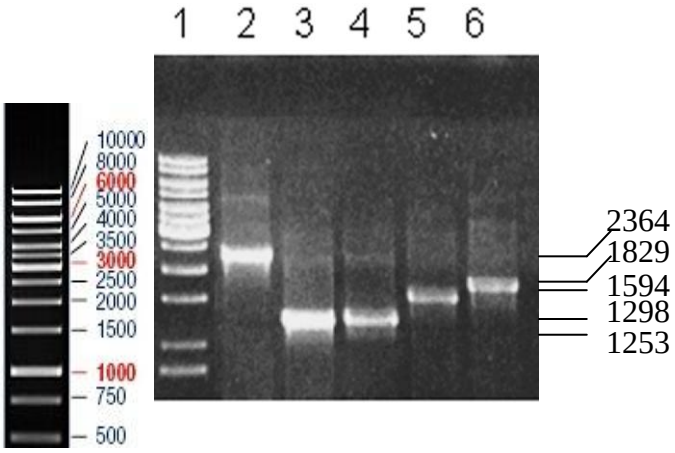

Figure 3. PCR amplifications of CMG457. Intergenic PCR product of pco operon of CMG457 plasmid pCMG457. Lane 1, 1 kb DNA ladder; lane 2, PCR product of *pcoAB* gene; lane 3, *pcoBC*; lane 4, *pcoCD*; lane 5, *pcoDR*; lane 6, *pcoRS*.

Primers used for the amplification of fragments of the Pco operon Table 1

Primer	Sequence (5' → 3')	**Position**[i]
CopA1[ii]	TCC ATA CAC TGG CAC GGC AT	558 – 577
CopA2[ii]	TGG ATC GGG TGA GTC ATC AT	1869 – 1888 (complement)
PcoB1	GTC GCC GGT TTG TTT ACC T	2123 – 2141
PcoB2	TTT TCG CCA TAT CGG ATG TT	2902 – 2921 (complement)
PcoC1	TTC TTA CAG GTG GCC TCG TT	3042 – 3061
PcoC2	CCG GTA ATA GGG TGC GTA TC	3356 – 3375 (complement)
PcoD1	TGG TAA TAT TTG GAT TGC CAT TT	3457 – 3479
PcoD2	CTG ACC TGA GAC GGA GCA A	4312 – 4330 (complement)
PcoR1	GGA CTG GTT GAG GAA GGC TA	4446 – 4465
PcoR2	CAC TCT TCT CTG ATC TCC AGG AC	5046 – 5068 (complement)
PcoS1	AAA ATT TCC CTG ACC ACA CG	5075 – 5094
PcoS2	GCC GGA CAT CGT ATT CGT AA	6255 – 6274 (complement)
PcoE1	CAT TAT GGC TGT CGC TTC AT	6713 – 6732
PcoE2	CAG CCT GCT GCT GAT GAA TA	7067 – 7086 (complement)

[i] In sequence of pco operon in *E. coli* plasmid pRJ1004 (GenBank accession number X83541)
[ii] underlined bases are different in pcoA gene of plasmid pRJ1004

Table 2. List of bacterial strains used in this study.

Strain Code	**Strain**	**Source**
CMG457	*Enterobacter* sp.	Centre for Molecular Genetics (CMG), culture stock
CMG457a	**Cured derivative of CMG457**	This study
CMG457b	Cured derivative of CMG457	This study
CMG457T	Transformant of CMG457	This study
XL1-Blue	*E. coli*	Standard (Recipient)
*p*PA173	Sub-cloned of the recombinant plasmid, *p*MU2385pco, which contains the entire Pco operon within a 9.0kb *Hind*III-*Bgl*II fragment. (6)	Nigel Brown, University of Birmingham, UK.

Conclusion

It is concluded that the complete Pco like operon of *E. coli* plasmid pRJ1004 is present in CMG457 plasmid (*p1*CMG457). It is suggested that the CMG457 has similar resistance mechanism against copper which has been reported earlier in E. coli plasmid *p*RJ1004 i.e., efflux system. Copper efflux pump encoded by *copA* gene was also reported on chromosomal DNA of *E. coli* strain K-12 (Outten et al., 2000). This has also revealed that the copper

resistant determinant was not species specific and is widely spread among bacteria isolated from entirely distinct geographical location.

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