

**IDENTIFICATION OF A PUTATIVE P-TYPE ATPase INVOLVED IN ZINC
AND CADMIUM RESISTANCE IN *Enterobacter* sp. YSU**

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IDENTIFICATION OF A PUTATIVE P-TYPE ATPase INVOLVED IN ZINC AND
CADMIUM RESISTANCE IN *Enterobacter* sp. YSU

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ABSTRACT

The Y-12 plant in Oak Ridge Tennessee was involved in manufacturing and storing nuclear weapon for the last 60 years of the 20th century. These activities led to the contamination of the nearby East Fork Poplar creek from which *Enterobacter sp.* YSU was isolated and screened for metal resistance genes. Three main previously identified metal resistance mechanisms are efflux, detoxification and sequestration. This bacterium was found to be resistant to several heavy metals including zinc, cadmium, mercury, copper, gold, chromium, silver and selenite. The goal of this study was to identify the gene encoding zinc and cadmium resistance in *Enterobacter sp.* YSU. Two mutants, EI24 and F24 were created using a transposon which disrupted a P-type ATPase believed to be responsible for these resistances. Minimal Inhibitory Concentrations (MICs) of the mutants were carried out to establish the phenotype of the mutants. These MICs revealed that the mutants were sensitive to toxic concentrations of zinc and cadmium while the wildtype bacterium was not. Gene rescue was carried out to rescue the disrupted gene and this was sequenced to determine its identity. BLAST identified the gene as a P-type ATPase with 97% similarity to a zinc/cadmium/mercury/lead translocating P-type ATPase in *Enterobacter cloacae*.

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TABLE OF CONTENTS

Abstract.....	(iii)
Acknowledgement.....	(iv)
CHAPTER I: INTRODUCTION.....	(1)
Y-12 plant, Oak Ridge Tennessee.....	(1)
<i>Enterobacter</i> sp. YSU.....	(2)
Metal resistance mechanisms.....	(2-10)
Hypothesis.....	(10)
CHAPTER II: MATERIALS AND METHODS.....	(12)
Growth media.....	(12)
Bacterial strains.....	(13)
Genomic DNA isolation.....	(13-14)
Plasmid DNA purification.....	(14-15)
Polymerized chain reaction.....	(15-16)
Genomic DNA digestion.....	(17)
Gel electrophoresis.....	(18)
Preparation of electrocompetent cells.....	(18-19)
Electroporation transformation.....	(19)
Replica Plating.....	(20)
Transposon mutagenesis.....	(20-21)
Rescue cloning.....	(21)
Ligation.....	(21)
DNA ligation precipitation.....	(22)
DNA Sequencing.....	(22-23)

BLAST analysis.....	(24)
CHAPTER III: RESULTS.....	(26)
MIC results.....	(27-31)
Gene rescue results.....	(32-37)
DNA sequencing.....	(38-48)
CHAPTER IV: DISCUSSION.....	(49-53)
CHAPTER V: REFERENCE.....	(54-68)

LIST OF FIGURES & TABLES

Figure1(A). Cadmium replica plating	(28)
Figure (B). Zinc replica plating.....	(29)
Figure 2. MIC chart.....	(30)
Figure 3. Genomic DNA digestion.....	(33)
Figure 4. F24 Plasmid DNA.....	(34)
Figure 5. EI24 Plasmid DNA.....	(36)
Figure 6. Blast results.....	(39)
Figure 7. EI24-F24 assembled sequence.....	(42)
Figure 8. DNA sequence alignment.....	(43)
Figure 9. VectorNTI map of P-type ATPase.....	(47)
Figure 10. Multiple protein sequence alignment.....	(48)
Table 1. Sequencing primers.....	(25)
Table 2. Plasmid sizes.....	(37)

LIST OF ABBREVIATIONS

EDTA.....	Ethylene diamine tetra acetic acid
HCl.....	Hydrochloric acid
μ L.....	Microliter
μ M.....	Micromolar
nm.....	Nanomolar
UV.....	Ultraviolet
BSA.....	Bovine serum albumin
dNTP.....	deoxyribonucleotidetriphosphate
TBE.....	Tris-Borate-EDTA
Zn.....	Zinc
Cd.....	Cadmium
Hg.....	Mercury
γ	Gamma
ATP.....	Adenosine triphosphate
GSH.....	Glutathione
NADP.....	Nicotinamide adenine dinucleotide phosphate
NADH.....	Nicotinamide adenine dinucleotide dehydrogenase
ATCC.....	American Type Culture Collection

CHAPTER I

INTRODUCTION

Y-12 Plant, Oakridge Tennessee

The Oakridge Y-12 plant now known as the Y-12 National Security Complex was originally constructed in 1943 as part of the Manhattan project. It is situated in Bear Creek Valley, east of the Oakridge Reservation in Anderson County Tennessee. During World War II, the Y-12 plant processed uranium to make the first atomic bomb. During the Cold war which started in the 1950s, it processed lithium using large amounts of mercury to make hydrogen bombs. However, at the end of the cold war, the mission changed from production of nuclear weapons to the production nuclear weapon components. Today, the Y-12 plant is involved in the production and recycling of nuclear weapon components and in the receipt, storage and safeguard of special nuclear materials as well as disposition of weapon components (40).

In light of the above mentioned past and present activities of this plant, it is apparent that it is also a key player in heavy metal contamination. Mercury was not well contained and large amounts of it (approx. 920, 000 kg) escaped and contaminated the nearby East Fork Poplar Creek (EFPC) as well as the surrounding environment. During the manufacturing of the atomic bombs in World War II, four S-3 ponds located near the Y-12 plant were used as disposal sites of acidic wastes contaminated with uranium as well as other heavy metals. The ponds were constructed without a covering or lining so as to allow some of the liquid wastes to be remediated as they passed through the soil. This

plan, however, was not effective and instead the wastes leached into the ground and in 2 EFPC. Eventually, the ponds were dredged, filled in, capped and converted to a parking lot (28).

***Enterobacter* sp. YSU**

This particular strain was obtained by the same group which isolated *Stenotrophomonas maltophilia* Oak Ridge Strain 02 (American Type Culture Collection {ATCC} #53510). This strain is capable of growing in toxic levels of mercury, cadmium, zinc, copper, and selenite (18, 28). Previously, the *Enterobacter* sp. YSU strain had been thought to be *S. maltophilia* OR02 strain but sequence analysis of 16s rDNA showed that it was in fact related to a strain of *Enterobacter* and further biochemical tests confirmed that it was actually an *Enterobacter*. This new strain was then called *Enterobacter* sp. YSU. According to previous studies done in my current lab, it is also resistant to different metals. The minimal inhibitory concentrations (MIC) of *Enterobacter* sp. YSU for mercury, cadmium, zinc, copper, gold, chromate, silver, arsenic and selenite were 0.07 mM, 0.24 mM, 0.8 mM, 3 mM, 0.05 mM, 0.4 mM, 0.08 mM, 14 mM and 40 mM respectively. The MIC of *S. maltophilia* O2 (ATCC #53510) for the same metals were 0.24, 0.33, 5, 5, 0.25, 7, 0.03, 14, and 40 Mm respectively (28).

Metal resistance mechanisms

Heavy metals can be toxic, not just to bacteria but even to higher forms of life. Some heavy metals generate reactive oxygen species that cause gene mutations making them hazardous to life. However, some of them are essential for life and these include copper, iron and zinc which are required in trace amounts. When their concentrations exceed the

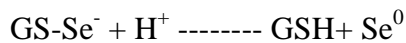
required levels, they become toxic to the cells (23).

There is no general mechanism for resistance to all heavy metal ions but from what is already known, heavy metal resistance genes in bacteria have been found on plasmids as well as in chromosomes. Three general resistance mechanisms found are efflux, enzymatic detoxification and sequestration (23). These mechanisms will be discussed in the next section.

Enzymatic detoxification

Enzymatic detoxification involves conversion of toxic metals into their less toxic forms, mediated by certain bacterial enzymes. A common example is mercuric reductase, MerA, which detoxifies mercury. It does so by transferring two electrons from NADPH to Hg^{2+} thus converting it to Hg^0 , its nontoxic form (30). The volatile Hg^0 then diffuses from the cell's environment into the atmosphere. The genes responsible for expression of mercuric reductase in gram-negative bacteria are arranged in the *mer* operon. This operon consists of *merT*, *P*, *C*, *A*, *D*, and *R* (30). The MerR protein is both an activator and repressor. It represses the *mer* genes in the absence of Hg^{2+} and activates expression of these genes in the presence of Hg^{2+} . The MerT and MerP together make up a transport system that brings the extracellular toxic mercury into the cell where it is reduced to its non-toxic form by MerA. Organomercurial lyase (MerB) confers mercury resistance to organomercurials. It contains three cysteine residues in its active site which are crucial in cleaving the C-Hg bond to release Hg^{2+} (60) before reduction.

Selenium (Se), a chemical element classified as either a non-metal or metalloid, also undergoes detoxification. The toxicity of selenium is due to reactive oxygen species⁻ which are produced during the reduction of selenite to elemental selenium as a result of the reaction of selenite and glutathione peroxidases. These damage membranes, DNA and other important bacterial components. Selenite also reacts with sulfhydryl groups of glutathione. The products of this reaction include selenogluthathione (GS-Se-SG), selenosupersulfide (GS-SeH), and hydrogenselenide (HSe) (34). The reduction process ends with GS-SeH, which is unstable and ultimately decays to nontoxic elemental selenium and glutathione (33) in the following reaction:



Sequestration of metals

This is performed by class II metallothioneins. These sequester cadmium as well as excess atoms of copper and zinc (61). Metallothioneins are cysteine-rich metal binding proteins with about 60 residues (35). It is the organization of the cysteine residues that helps the metallothioneins bind essential metal ions. The sulfhydryl group interacts with the metal ions, binding to them in the process as in the case of *Synechococcus* cyanobacterium (36). The metals whose transport is regulated in this way include cadmium, copper, zinc, lead, nickel and arsenate (37). Heat shock genes are also normally expressed by bacteria as a result of environmental stress. An example is GroEL, a protein that when complexed with GroES helps proteins to renature and fold properly after denaturation. These two are always arranged in an operon and are induced by stress

factors such as high temperatures. (38)

Efflux pumping

Efflux pumping is a system developed by most bacteria to extrude unwanted toxic substances from cells. It is the major detoxification mechanism used by bacteria, some plants (14) as well as yeast (63). Metal efflux in bacteria can be mediated by the following: resistance nodulation cell-division (RND)-type exporters, cation diffusion facilitators or P- type ATPases (29).

RND-type exporters

Members of this superfamily are involved in the transport of various substances. They transport heavy metals, hydrophobic compounds, amphiphilic compounds, nodulation factors as their name suggests, and also work with SecB in protein transport (31). This section mainly deals with the members that mediate heavy metal efflux. The suggested general mechanism of action by all RND transporters includes a protonation/deprotonation cycle that couples substrate binding and release to the exergonic proton import reaction. The initially RND protein-bound substrates are subsequently forwarded to the other components of the efflux system for further transport and ultimately, export (2). Generally, RND-mediated heavy metal efflux is composed of three subunits.

The first one to be identified was the CzcABC metal efflux pump found in *Ralstonia metallidurans* (*R. metallidurans*) which is now known as *Cupriavidus metallidurans* (*C. metallidurans*). This pump is mainly responsible for resistance to cadmium, zinc and

cobalt as suggested by its name. It is composed of three main proteins CzcA, an inner membrane protein CzcB, a periplasmic protein and CzcC an outer membrane protein (29). This pump maintains high-metal resistance against the three mentioned metals, by active cation efflux driven by the proton motive force (2). The *cnrABC* was identified in the same bacterium. This pump uses the same mechanism as CzcABC to pump out nickel metal cations (31). The CzcABC exports metals from the periplasm directly by funneling periplasmic cations into a transenvelope efflux system or indirectly by the combined effect of a CBA transport efflux system (31).

E. coli has several RND-type exporter genes but the only one specifically involved in metal resistance is the *cusCFBA*. This is involved in copper and silver metal resistance (6, 31). CusA, the inner membrane protein interacts with the membrane fusion protein CusB and the outer membrane protein CusC. The periplasmic protein CusF binds copper and silver and thereafter transfers it to the membrane fusion protein (25) which in turn transfers it to the outer membrane protein ultimately, depositing it out of the cell. The *cusR* and *cusS* genes, located upstream of *cusCFBA*, encode the CusR/ CusS system which regulates gene expression. This system is mainly involved in cellular copper homeostasis and *cusCFBA* expression (6, 27).

In *Salmonella*, GesABC has been identified as a gold efflux pump which is encoded by *gesABC*. GesB is the inner membrane RND transporter, GesA is the membrane fusion protein and GesC is the outer membrane fusion protein (OMP) (26). As opposed to CusCFBA, recent studies done using gene manipulation mechanisms revealed that GesABC has broad substrate specificity for other RND-type system regulated metals

(24).

Cation Diffusion Facilitators (CDF)

In contrast to RND systems, cation diffusion facilitators are single sub-unit transporters, comprised of a number of membrane bound proteins and occurring in all life domains (7, 8). They transport five main substrates: Zn^{2+} , Co^{2+} , Ni^{2+} , Cd^{2+} and Fe^{2+} . CDF-mediated transport is driven by a chemiosmotic gradient formed by protons or potassium (9, 11). These proteins were first identified in *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Wautersia metallidurans* (*W. metallidurans*) (39) and they were able to transport Cd^{2+} , Ni^{2+} and Fe^{2+} . The zinc and cadmium transporting CDF protein was called CzcD, and the mechanism of action was established as electroneutral antiport because positive metal ions were being transported out of the cytoplasm into the periplasm in exchange for H^+ or K^+ (9). It was also found to regulate expression of *czcABC* but it is also capable of mediating a somewhat low degree of $Zn^{2+}/Co^{2+}/Cd^{2+}$ resistance on its own (12). However, in *E. coli* in which it was heterologously expressed, it exported only zinc and cadmium. It was also not electroneutral since the ratio of zinc to H^+ was 1:1 (22) but was driven by a proton motive force. The CzcD system also mediates efflux of Zn^{2+} , Co^{2+} and Cd^{2+} in *Bacillus subtilis* (*B. subtilis*) (31).

Further analysis on the recently completed genomic sequence of *W. metallidurans* revealed two more CDF encoding genes. These were found to be carried on the bacterial chromosome rather than a megaplasmid. These are *dmef* (divalent metal efflux) and *fieF*

(Ferrous Iron Efflux). Gene expression experiments done in *E. coli* showed that these two genes encode CDF proteins that mediate zinc resistance and increase tolerance towards iron (30). However it was also shown that ZitB, the CDF protein mediating tolerance in *E. coli*, was more effective at it than these novel CDF protein encoding genes (39). ZitB and CzcD from *B. subtilis* and *R. metallidurans* are driven by a potassium gradient or a proton motive force (13, 9). When *E. coli* strains were grown with a high concentration of iron, they had diminished growth but the expression of *fieF* in one of the strains increased tolerance to iron and there was an increase in growth (39).

P-type ATPases

Members of this superfamily are found in all the kingdoms of life and their main substrates are H^+ , Na^+ , K^+ , Mg^+ , Ca^{2+} , Cu^+ , Zn^{2+} and Cd^{2+} (15). Like CDF, they are single sub-unit transporters (65). They are all driven by energy derived from ATP hydrolysis and their functions include both importing and exporting. The focus of this study is on the exporting P-type ATPase with respect to heavy metal detoxification but the import systems are also important as in the case of importing macroelements such as magnesium. P-type ATPases have three main domains: The A domain, P domain and N domain. The N domain contains the nucleotide binding site and forms a seven stranded antiparallel beta sheet with two additional beta strands (55). This domain is involved in ATP binding. The A-domain, meaning actuator domain, also known as cytoplasmic transduction domain is involved in transposing energy from ATP hydrolysis for cation transport in the transmembrane domain. The P domain contains aspartic acid which is phosphorylated during the reaction cycle (59).

Additionally, the N-terminal regions of some ATPases such as CopB ATPase have a copper binding domain with three repeats of the consensus sequence Met-Xaa-His-Xaa-Xaa-Met-Ser-Gly-Met-Xaa-His-Ser. The N-terminal region of CopA constitutes the conserved motif, Gly-Xaa-Thr-Cys-Xaa-Xaa-Cys, also found on the cadmium-transporting ATPase, CadA, which transports cadmium from the cytoplasm to the periplasm (65). The hydrophobic domain of both CopA and CopB contain a proline residue which is conserved in all P-type ATPases (66). Members of the family that mediates heavy metal transport are characterized by the conserved proline residue which is heavily flanked by cysteine residues (16). These are commonly called Cpx-type ATPases. The two types of CPx-type ATPases are discussed below.

Cu-CPx-type ATPases which export Cu^+ and Ag^+ (4, 5). These have been identified in gram-negative and gram-positive bacteria including *E. coli* and *B. subtilis* (3). **Zn-CPx-type ATPases** are involved in the export of Zn^{2+} , Cd^{2+} , and Pb^{2+} . CadA, a member of this family is involved in cadmium and zinc resistance and was found in *Staphylococcus aureus* (17). It was later found in other bacteria as well, including *S. maltophilia* and *E. coli*. However, these Cpx-ATPases are not as widespread as the Cu-CPx-ATPases (31).

The substrate specificity of Cpx-type ATPases includes Cu^+/Ag^+ and/or $\text{Zn}^{2+}/\text{Cd}^{2+}/\text{Pb}^{2+}$ and they are mostly involved in export although import can also be accomplished (31). For instance, two types of Cu-CPx-type ATPases were found in

Enterococcus hirae (*E. hirae*). CopA, involved in import of copper and CopB which mediates efflux from cytoplasm to periplasm (19, 65). From these findings, it is clear that an efflux system could change into an uptake system and vice versa, depending on the environment of the cell, because the ultimate goal is to survive. Bacteria need copper but only as a trace nutrient since too much of it would cause oxidative stress. Hence they need both an uptake and efflux system.

Other heavy metal exporters

There are other exporters that do not belong to the three main categories mentioned above. These are ChrA-like proteins which belong to the CHR protein family. They are involved in detoxification of oxyanion chromate (21, 20), driven by chemiosmotic gradient. The others are NreB and CnrB which export nickel and probably use a proton motive force since experimental evidence has proven that they lack ATP binding sites (31). The objective of this study is to use transposon mutagenesis to identify metal resistance genes in *Enterobacter sp.* YSU. Studying these identified genes in detail may reveal why a particular resistance mechanism is more effective than another.

Hypothesis

Transposon mutagenesis was used to obtain two mutants designated EI24 and F24. These mutants failed to grow in toxic concentrations of zinc and cadmium compared to the wildtype. I propose that cadmium and zinc resistance in *Enterobacter* sp. YSU encodes a P-type ATPase, RND or CDF. This is based on the fact that these are the three efflux systems that are known to mediate divalent heavy metal transport.

CHAPTER II

MATERIALS AND METHODS

Growth media

Lennox LB broth (Fischer Scientific, Fair Lawn, NJ) contained 10 g of tryptone, 5 g of yeast extract and 5 g of NaCl per liter. When required, it was supplemented with 1.6% agar (Amresco Inc., Solon, OH) and/or 50 µg/ml of kanamycin (Amresco, Solon, OH).

R3A-Tris (28) contained 1 g of yeast extract (Fisher Scientific, Fair Lawn, NJ), 1 g of Difco Proteose peptone no.3 (Difco Laboratories, Sparks, MD), 1 g of Casamino acids (Amresco, Solon, OH), 1 g of glucose (Amresco, Solon, OH), 1 g of soluble starch (Difco Laboratories, Sparks, MD), 0.5 g of sodium pyruvate (Fisher Scientific, Fair Lawn, NJ), 10 ml of Tris PH 7.5 (Amresco, Solon, OH), 0.1 g of MgSO₄·7H₂O (Fisher Scientific, Fair Lawn, NJ) per liter. When needed, it was supplemented with 1.6% agar (Amresco Inc., Solon, OH).

SOC broth contained 20 g of tryptone, 5 g of yeast extract, 2ml of 5M NaCl, 2.5 ml of 1M KCl, 10 ml of 1 M MgCl₂, 10 ml of 1 M MgSO₄ and 20 ml of 1 M glucose per liter and filter-sterilized. When required, media was supplemented with 50 µM HgCl₂ (Fisher Scientific, Fair Lawn, NJ), 700 µM ZnCl₂ (Fisher Scientific, Fair Lawn, NJ) and 500 µM CdCl₂ (Fisher Scientific, Fair Lawn, NJ).

Bacterial strains

E. coli strain ECD100D *pir* 116 (Epicentre, Madison WI) - *F* *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*dlacZAM15* Δ *lacX74* *recA1* *endA1* *araD139* Δ (*ara*, *leu*) 7697 *galU* *galK* λ -*rpsL* (*Str^R*) *nupG* *pir-116(DHFR)* - contain the *pir* gene product for replication of plasmids with the R6Ky replication origin.. This strain was used for gene rescue.

Enterobacter sp. YSU is a multimetal resistant strain from Oak Ridge TN (28). It is resistant to salts of mercury, silver, copper, selenium, arsenic and zinc. The zinc-sensitive and cadmium-sensitive mutants, F24 (85) and EI24 were generated from *Enterobacter* sp. YSU by transposon mutagenesis.

Genomic DNA isolation

This is a multi-stage process for purification and isolation of genomic DNA, and was performed using the Wizard genomic isolation kit from Promega (Promega Corporation, Madison, WI). 1 ml of overnight culture was centrifuged for two minutes at 15000xg. The supernatant was poured off by decanting, the cells were resuspended in 600 μ l of nuclei lysis solution, incubated at 80°C for 5 minutes and cooled to room temperature. 3 μ l of RNase solution [4mg/ml] (Promega Corporation, Madison, WI) was added to the lysate which was then mixed by inversion 2 to 5 times followed by 15-60 minutes of incubation at 37°C. The lysate was cooled down to room temperature and 200 μ l of protein precipitation solution was added and vortexed for 20 seconds followed by incubation on ice for 5 minutes. The mixture was centrifuged at 15000 xg for 3 minutes.

The supernatant containing the DNA was transferred to a clean microcentrifuge tube containing 600 µl of isopropanol. This was mixed by inversion until thin strands of DNA formed a visible mass after which the mixture was centrifuged for 2 minutes at 14000 xg. Since DNA is insoluble in alcohol, it sticks together and readily forms a pellet upon centrifugation. The supernatant was poured off and the rest drained on clean absorbent paper. Seventy percent ethanol was then added and the tubes were gently inverted up and down several times to wash the pellet. After centrifuging at 14000 xg for 2 minutes, the ethanol was carefully decanted and the tubes were drained on absorbent paper and air-dried for 15 minutes. Finally, the DNA was rehydrated with 100 µl of DNA rehydration solution and incubated overnight at 4°C.

Plasmid DNA purification

Plasmids were purified using Promega's Wizard[®] Plus SV MinPrep DNA purification kit. 10 ml of bacteria containing a plasmid were harvested by centrifugation for 5 minutes at 10000 x g. The supernatant was poured off and the inverted tubes blotted on absorbent paper to remove excess media. 250 µl of Cell Resuspension solution (50 mM Tris-HCl, 10 mM EDTA, 100 µg/ml Rnase A) was added to the cells to resuspend them. Complete resuspension was ensured by vortexing. Thereafter, resuspended cells were transferred to microcentrifuge tubes. After this step, it is necessary not vortex anymore but just invert the tubes if mixing is required to avoid chromosomal DNA shearing and contamination. 250 µl of cell lysis solution (0.2 M NaOH, 1% SDS) were added to the resuspended cells and mixed by inverting 4 times. 10 µl of alkaline protease solution (250 µg) were added

and the tubes inverted 4 times followed by incubation for 5 minutes at room temperature. Next, 350 μ l of neutralization solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate, 2.12 M glacial acetic acid) was added and mixed with the lysate by inverting the tube 4 times. The bacterial lysate was then centrifuged at 14000 \times g for 10 minutes at room temperature.

The spin columns were prepared by inserting each column into a 2 ml collection tube. The cleared lysate was then transferred to the spin column by decanting. The supernatant was centrifuged at maximum speed for 1 minute at room temperature. The spin column was removed and the flow through in the collection tube discarded, then the spin column was reinserted. 750 μ l of Column Wash (162.8 mM potassium acetate, 22.6 mM Tris-HCl pH 7.5, 0.109 EDTA PH 8.0, 58% ethanol) was added to the spin column and centrifuged at top speed for 1 minute at room temperature. The flow through was discarded and this step was repeated but with 250 μ l of Column Wash followed by centrifuging for 2 minutes. The spin column was carefully transferred to a sterile 1.5 ml microcentrifuge tube. DNA was eluted by adding 100 μ l of nuclease free water to the spin column and centrifuging at maximum for 1 minute at room temperature. The eluted DNA was stored at -20°C.

Polymerized Chain Reaction (PCR)

The polymerase chain reaction was used to amplify DNA regions of known sequences made possible by its ability to denature double stranded DNA molecules and then renature the complimentary single strands in a controlled manner. The three main steps of

the PCR reaction are DNA denaturation, primer-template annealing and DNA elongation, achieved by the use of DNA polymerase (41). In this case GoTaq DNA polymerase (Promega Corporation, Madison, WI) was used to amplify a short segment of the P-type ATPase that could not be obtained by gene walking. The components of the PCR were 2x GoTaq DNA polymerase (0.025 µg), EI24 R1 primer (0.00625 µg) and F24 R3 primer (0.00625 µg) from table 1, Plasmid DNA (0.001 µg) and nuclease free water (10 µl). GoTaq DNA polymerase contains bacterial derived *Taq* DNA polymerase, dNTPs, MgCl₂ and a green loading dye. The PCR was carried out in the Thermal Cycler as follows: 95°C for 2 minute, 95°C for 1 minute (denatures DNA), 55°C for 1 minute (primer annealing), 72°C for 30 minute (extension), repeat step two 34 times, 72°C for 10 minutes, hold at 10°C.

PCR reactions were cleaned up using the QIAquick PCR purification kit (Qiagen Sciences, MD). 1 volume of PCR product was mixed with five volumes of buffer PB which contains 5.0 M guanidine hydrochloride (GuHCl) and 30% isopropanol. This was mixed by pipetting up and down, then loaded into MinElute columns and a vacuum applied. The PCR products were retained on the column, while the buffer, primers, deoxyribonucleotides and polymerase washed through. The remaining contaminants were washed through a 750 µl column wash step followed by a second 500 µl column wash using buffer PE. The DNA was then eluted with 10µl buffer EB (10 mM Tris-HCl, pH 8.5).

Genomic DNA digestion

The genomic DNA was partially digested using *Bfu*CI (New England BioLabs Inc., MA) which was 4000 U/mL in stock. It yields 'GATC compatible sticky ends (44). This entailed making an initial mixture of 1X BSA [10 mg/ml] (New England BioLabs Inc., MA), 1X Buffer 4 [50 mM potassium acetate, 20mM Tris-acetate, 10mM magnesium acetate, 1mM, dithiothreitol] (New England BioLabs Inc., MA), 7 µl nuclease free water (New England BioLabs Inc., MA) and 1 µl *Bfu*CI. Then a second mixture containing 15.2 µl of genomic DNA, 1X buffer 4 and 1X BSA was made. The enzyme was diluted by a factor of 200=4/200 U in the reactions. Digestion mixtures were incubated at 37°C for 20 minutes, and then at 80-85°C to inactivate the enzymes. This was carried out in the Eppendorf Master Cycler. Other restriction enzymes used were *Sac* I- 10 µl of genomic DNA, 2 µl of 10X buffer 1, 100 µg/ml BSA, 1 µl *Sac* I and 5 µl of nuclease free water. Heat inactivation at 65°C for 20 minutes; *EcoR* I- 10 µl of genomic DNA, 4 µl of 10X *EcoR* I buffer, 1 µl of *EcoR* I and 5 µl of water and heat inactivation at 65°C for 20 minutes; *EcoR* V- 10 µl of genomic DNA, 2 µl of 10X buffer 3, 100 µg/ml BSA, 1 µl of *EcoR* V and 5 µl of nuclease free water and heat inactivation at 80°C; *Nco* I- 10 µl genomic DNA, 4 µl of 10X buffer 3, 1 µl of *Nco* I and 5 µl of nuclease free water and heat inactivation at 65°C for 20 minutes; *Aat* II- 10 µl of genomic DNA, 4 µl of 10X buffer 4, 1 µl of *Aat* II and 5 µl of nuclease free water. All the buffers and BSA and nuclease free water were obtained from New England BioLabs Inc., MA. The digestion reactions were incubated overnight and then the restriction enzymes were inactivated at the recorded temperatures.

Gel electrophoresis

DNA samples were frequently separated on a 0.8% agarose gel. The gels were made using 1.04g of agarose (Fisher Scientific, Fair Lawn, NJ) dissolved in 130 ml of Tris Borate EDTA(TBE) buffer [Tris 0.089 M, Borate 0.089 M, EDTA 0.002 M,], (Amresco, Solo, OH). The solution was dissolved in a microwave for 2 minutes with periodic swirling to ensure the agarose was uniformly distributed. It was then cooled down to about 50°C then poured into the casting trays and the combs inserted. The combs were gently pulled out after 30 minutes when the gels were solidified. Once the gel was placed in the gel box it was submerged in 1X TBE. The DNA samples were mixed with an appropriate volume of EZ Vision (Amresco, Solon, OH) dye and loaded in to the wells. The gel was then run at 100 V until the blue dye which is equivalent to about 50 base pairs reached the end of the gel. A picture of the gel was then taken under UV light using UltraCam Imaging Systems (Ultra-Lum, Inc. Claremont, CA).

Preparation of electrocompetent ECD pir/pir 116

For the purposes of electroporation, ECD pir (low copy) or ECD pir 116 (high copy) were prepared. An overnight 5 ml LB culture was prepared. The next day, these were transferred to 160 ml of LB and incubated in the 37°C shaker until they reached an OD of 0.4-0.6 at 600 nm. These were chilled on ice for about 15 minutes and then transferred to pre-chilled, sterile 80 ml centrifuge tubes. They were spun at 5000 rpm in the pre-chilled centrifuge at 4°C to harvest the cells. After pouring off the supernatant, the cells were washed twice with pre-chilled, sterile water in equal volumes. For each wash, a small volume was added to the pellet and shaken to resuspend the cells. The tubes were then

filled up to 80 ml with water followed by centrifuging at 5000 rpm for 15 minutes at 4°C. After the final wash, 200 µl of pre-chilled 10% glycerol was added to the pellet, mixed and divided into microcentrifuge tube. The cells were centrifuged at 4°C in a pre-chilled microcentrifuge for 10 minutes at 14000 rpm, the supernatant discarded and the cells were resuspended in 250 µl of ice cold 10% glycerol and stored at -80°C.

Electroporation Transformation

The electroporation cuvettes and GeneMate microcentrifuge tubes (BioExpress, Kaysville, UT) were chilled on ice. The electrocompetent cells were thawed on ice and the electroporation machine turned on. 40 µl of electrocompetent cells were placed in the microcentrifuge tube and 2 µl of the ligation reaction added. The mixture was pipetted into the cuvette and tapped to the bottom of the tube. It was then shocked at 25 µF, 200 ohms and 2.5 kV. Immediately, the cells were resuspended in 960 µl of SOC media and mixed by pipetting up and down. They were incubated in the 37°C shaker for 45 minutes to give the cells time to express antibiotic resistance on the plasmid (43). Finally, 100 µl of the cells were plated on LB plates containing kanamycin and incubated overnight at 37°C.

Replica plating

Bacterial colonies were spotted on an R3A-tris agar plate in the form of a grid and allowed to grow overnight at 30°C. A sterile velveteen cloth was placed over a block,

fuzzy side up. The R3A agar plate containing transformants with inserts was then inverted and gently pressed onto the velveteen cloth. A second, fresh plate R3A agar was inverted on the velveteen and the colonies transferred by gently pressing it over the cloth. The velveteen cloth was discarded, the block cleaned with 70% ethanol and a fresh cloth placed over it. The colonies were then transferred from the second chloramphenicol plate to the fresh cloth then metal plates were inoculated with bacteria by gently stamping them on the block. The metal plates were incubated at 30°C overnight. The minimal inhibitory concentration (MIC) of both *Enterobacter* YSU and its mutants were established by observing at which concentration of either zinc or cadmium they stopped growing. The MICs were then plotted on a chart.

Transposon mutagenesis

To generate the EI24 and F24 mutants, transposon mutagenesis was carried out using the EZ-Tn5 <R6K_{ylori}/KAN-2> Tnp transposome kit (Epicentre technologies Madison, WI). 0.4 µl of EZ-Tn5 Transposome was mixed with 40 µl of electrocompetent cells and the mixture transformed by electroporation. Immediately, 960 µl of SOC media was added to the electroporated cells and mixed by pipetting up and down. The transformation was then incubated at 30°C for 45 minutes in a New Brunswick Scientific shaker (Edison, NJ). Transformants were then selected on LB plates supplemented with 50 µg/ml kanamycin. Colonies that grew were spotted on a grid using fresh kanamycin plates and incubated at 37°C overnight. Finally, they were screened for zinc and cadmium

sensitivity by replica plating them on R3A-tris agar plates supplemented with CdCl₂ ZnCl₂.

Rescue cloning from transposed genomic DNA

The selected mutants were cultured overnight at 30°C in 5 ml of LB supplemented with 50 µg/ml kanamycin followed by genomic DNA purification as described previously. The purified genomic DNA was then fragmented with the restriction enzymes mentioned above. The fragments were then subjected to self-ligation.

Ligation

The components of a ligation mixture were 2 µl of T4 DNA ligase (New England *BioLabs* Inc., Beverly, MA), 50 µl of 10X T4 DNA ligase buffer (New England *BioLabs* Inc. Beverly, MA) containing 10mM ATP, 15 µl of digested genomic DNA and 433 µl of nuclease free water to bring the total volume to 500 µl. The ligation was then incubated overnight at 4°C.

DNA Ligation precipitation

A tenth of a volume (50 µl) of 3 M sodium acetate, pH 5.2 and two volumes of 95% ethanol was added to the ligation reaction and incubated at -20°C for ten minutes. The

ligation was then centrifuged at top speed for ten minutes in an Eppendorf centrifuge. The supernatant was poured off and then 300 μ l of 70% ethanol was added. The tube was gently inverted twice and centrifuged for five minutes. The supernatant was poured off and the remaining liquid was drained by blotting on a paper towel. The pellet was then dried in the CentriVap (Labconco Corporation, Kansas City, MO). The dried pellet was finally resuspended in 10 μ l of nuclease free water and 2 μ l of this was used in an electroporation transformation.

DNA sequencing

Sequencing was performed using GenomeLab™ Dye Terminator Cycle sequencing according to the instructions in the Quick Start Kit (Beckman Coulter, Inc. Fullerton, CA). The DNA volume to be used was calculated based on size and concentration of the DNA as outlined in the kit instructions. The sequencing reaction was performed by mixing the appropriate amounts of DNA, nuclease free water, heating for 1 minute at 96°C and then adding the forward and reverse primer (Table 1) obtained from Epicentre Technologies and the Dye Terminator Cycle Sequencing (DTCS) Quick Start Master Mix. For the EI24 clones resulting from an *Nco* I digest, 4.8 μ l of plasmid DNA was mixed with 5.2 μ l of nuclease free water. For F24 clones resulting from *Nco* I digestion, 8.0 μ l of plasmid DNA was mixed with 2 μ l of nuclease free water. For EI24 clones resulting from *Sac* I digestion, 8.7 μ l was mixed with 1.3 μ l nuclease free water. For F24 clones resulting from *Aat* II, 5.3 μ l of plasmid DNA was mixed with 4.7 μ l of nuclease free water. All the sequencing reactions contained 8 μ l of DTCs and 2 μ l of each primer.

The contents were mixed in thin walled 200 μ L PCR tubes (BioExpress, Kaysville, UT) and incubated in an Eppendorf Master Cycler according to the following program: 96°C for 20 seconds, 50°C for 20 seconds and 60°C for 4 minutes for 30 cycles. The holding temperature was 4°C. DTCS contains DNA polymerase, pyrophosphatase buffer, dNTPs and dye terminators.

The sequencing reactions were cleaned up according to the Beckman Coulter protocol. 5 μ l of Stop solution (3M sodium acetate, pH 5.2; 100 Mm Na₂-EDTA, pH 8.0 and 20 μ g/mL glycogen) was mixed with the sequencing reaction in new tubes. DNA precipitation was done by adding 60 μ l of ice cold 95% ethanol centrifuging at 14000 rpm at 4°C for 15 minutes. The pellet was rinsed twice with 70% ethanol by centrifuging at 14000 rpm for 2 minutes. The pellet was then air dried in the CentriVap DNA concentrator (Labconco Corporation, MO) for 10 minutes and then resuspended in 40 μ l of Sample Loading Solution. Finally, the DNA was analyzed using the Beckman Coulter CEQ 2000XL DNA analysis system (Fullerton, CA) by Mr. Ed Budde.

Molecular Evolutionary Genetics Analysis version 5 (MEGA5) and ClustalX2 were used to build sequence alignments (85, 86). Genedoc was used to edit the sequence (87) and ContigExpress from the VectorNTI (Life technologies) was used to assemble all the sequences into one large continuous sequence.

BLAST analysis

Basic Local Alignment Search Tool (BLAST) is used to compare nucleotide and protein sequences so as to identify gene or protein families to which novel sequences

belong to. The sequences in question are compared to a library of sequences at the National Library of Medicine (NLM) and sequences with a high percentage identity may be grouped together. The query sequence is entered in FASTA format and the programs blastn (nucleotide) or blastp (protein) are chosen. Alternatively, the accession number or gi could be used. After choosing the appropriate set for the sequence in question, clicking the BLAST button is the final step (58).

Table 1. Primers used for DNA sequencing

Primer	Sequence	Concentration (nm)
Zn ATPase F	5'-CCAGCGCAATCCCAATGTTC-3'	38.2
Zn ATPase R	5'-ATTAGAGCAGGCCGGGCAGA-3'	32.6
Zn EI24 R1	5'-AAGGCTTCCACCATCGGCATA-3'	33.5
Zn F24 F1	5'-GCCAACATCCGCCAGAACATT-3'	33.8
Zn F24 R1	5'-TCTGCCCGGCCTGCTCTAAT-3'	31.2
Zn EI24 F1	5'-CAACGCTAATGCAATGGTAA-3'	29.6
Zn F24 R2	5'-TCTATCAGCCTGAGGATGCG-3'	25.2
Zn F24 F2	5'-GACGGTGCTGGTTACGGCGAA-3'	25.0
Zn F24 F3	5'-GCTGCTGATCGGCTGTCCGTG-3'	29.2
Zn F24 R3	5'-TTCGCCGTAACCAGCACCGTC-3'	35.4
Zn F24 F4	5'-CCTCGTCACCACGCTGCT-3'	34.8
Zn F24 R4	5'-CAGCGTGGTGCCGATAAAC-3'	34.6
Zn F24 F5	5'-GGTTATTCGTGGTCAGCGTG-3'	33.5
Zn F24 F6	5'-CTGGAGCAAATCAACCAC-3'	31.3
TRAP F2	5'-CCATTTTGCTGGCGCTG-3'	32.1
TRAP NcoI R1	5'-ACCAACAGCGAAATACCTGC-3'	33.8
TRAP F1	5'-AACGCCTGCTGTTTGGCTCGC-3'	29.0
Zn F24 R5	5'-GTGGTTGATTTGCTCCAG-3'	25.4
Zn F25 R6	5'-ATAACCCGCTTTGCTGACG-3'	27.3
R6KAN-2 R	5'-CTACCCTGTGGAACACCTACATCT-3'	50000

CHAPTER III

RESULTS

Transposon mutagenesis was used to generate the F24 and EI24 mutants. Transposons insert into the bacterial genome, disrupting the function of the interrupted gene and its neighbors. The current study used a transposon which lacks a transposase gene. It contained an RK6 γ replication origin and a kanamycin resistance gene DNA segment that was flanked by two identical inverted repeat/mosaic ends. Purified transposase added to the transposome construct bound to the mosaic ends and catalyzed random insertion when introduced into a bacterial host delivered by electroporation. The Tn5TM R6K γ ori/KAN-2>Tnp TransposomeTM, is a very stable DNA complex, maintaining integrity throughout the transformation process (73).

F24 mutant was previously generated in another study (85). To generate the EI24 mutant 880 transposome transformed *Enterobacter* sp. YSU colonies were screened by replica plating onto R3A-Tris plates supplemented with 100 μ M of zinc. One zinc-sensitive mutant was streaked out and grown overnight at 30°C. Three individual colonies were streaked out on fresh LB plates supplemented with kanamycin (LB-Kan) plates and grown overnight at 30°C. To verify that these isolated colonies were mutants, they were spotted on an LB-kan plate and then replica plated once again on R3A-tris agar plates supplemented with zinc and cadmium. A confirmed mutant colony was grown in LB-kan overnight and frozen as a glycerol stock at -80°C.

MIC results

Frozen stocks of *Enterobacter* sp. YSU and the mutants were streaked out on LB-kan plates. Three colonies of each were spotted on LB agar plates and grown overnight at 30°C. They were then replica plated onto R3A-tris plates supplemented with 100 µM, 200 µM, 300 µM, 400 µM, 500 µM, 600 µM, 700 µM and 800 µM zinc or 0.015 mM, 0.020 mM, 0.025 mM, 0.05 mM, 0.10 mM, 0.20mM and 0.25mM cadmium. The MIC of EI24 and F24 in zinc was 200 µM compared to 700 µM of the wildtype (Fig. 1). In cadmium, the MIC of the EI24 and F24 was 0.015 mM compared to 0.5 mM of the wildtype (Fig.1). From these results, it is apparent that these two mutants are sensitive to zinc and cadmium.

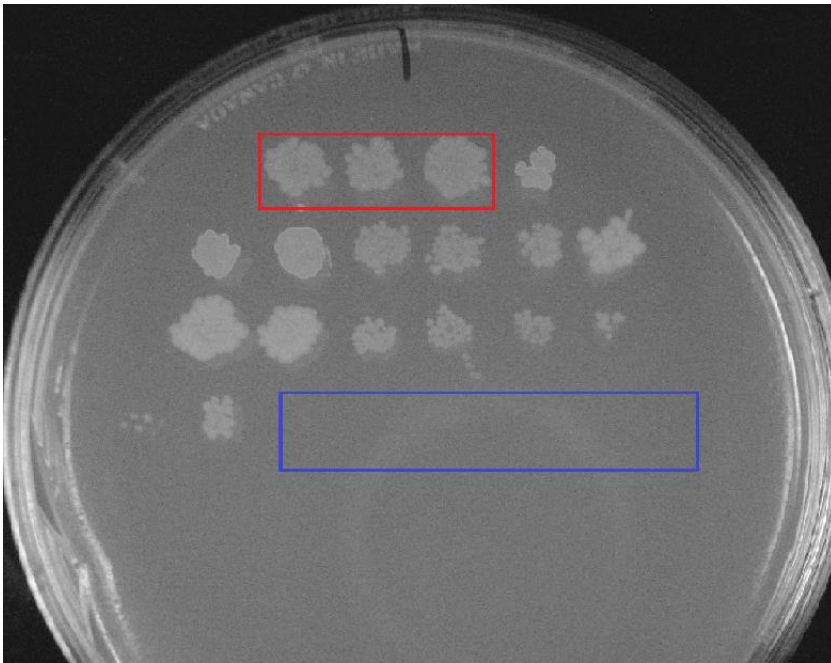


Fig.1(A). 0.05 mM cadmium replica plating result with *Enterobacter sp.* YSU, EI24 and F24 mutants. MICs were determined by spotting colonies on LB agar plates, growing them overnight and replica plating them onto plates containing different metal concentrations. Red box indicates wildtype *Enterobacter sp.* YSU and the blue box indicate the EI24 and F24 mutants.

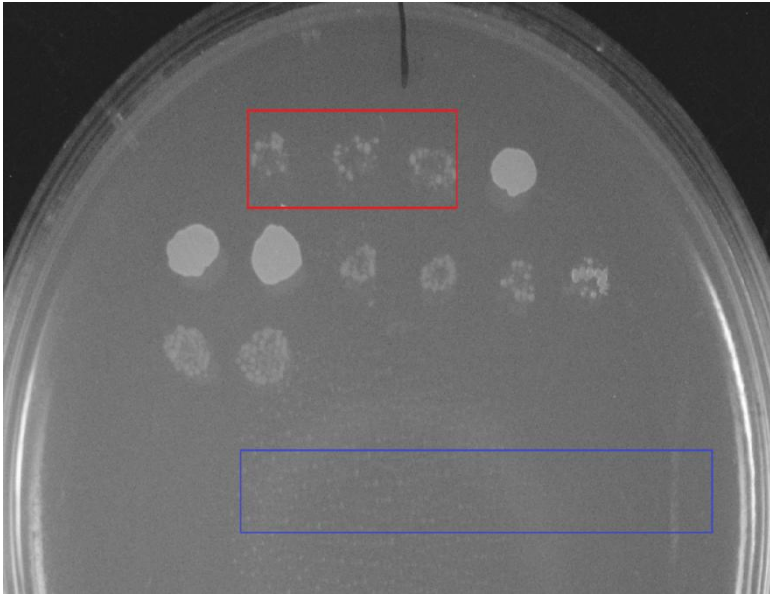


Fig. 1(B). Zinc replica plating at 600 μM . Red box encloses the wildtype *Enterobacter* sp. YSU. Blue box encloses the space where the mutant spots should be. The other colonies are potential mutants and have no effect on this study.

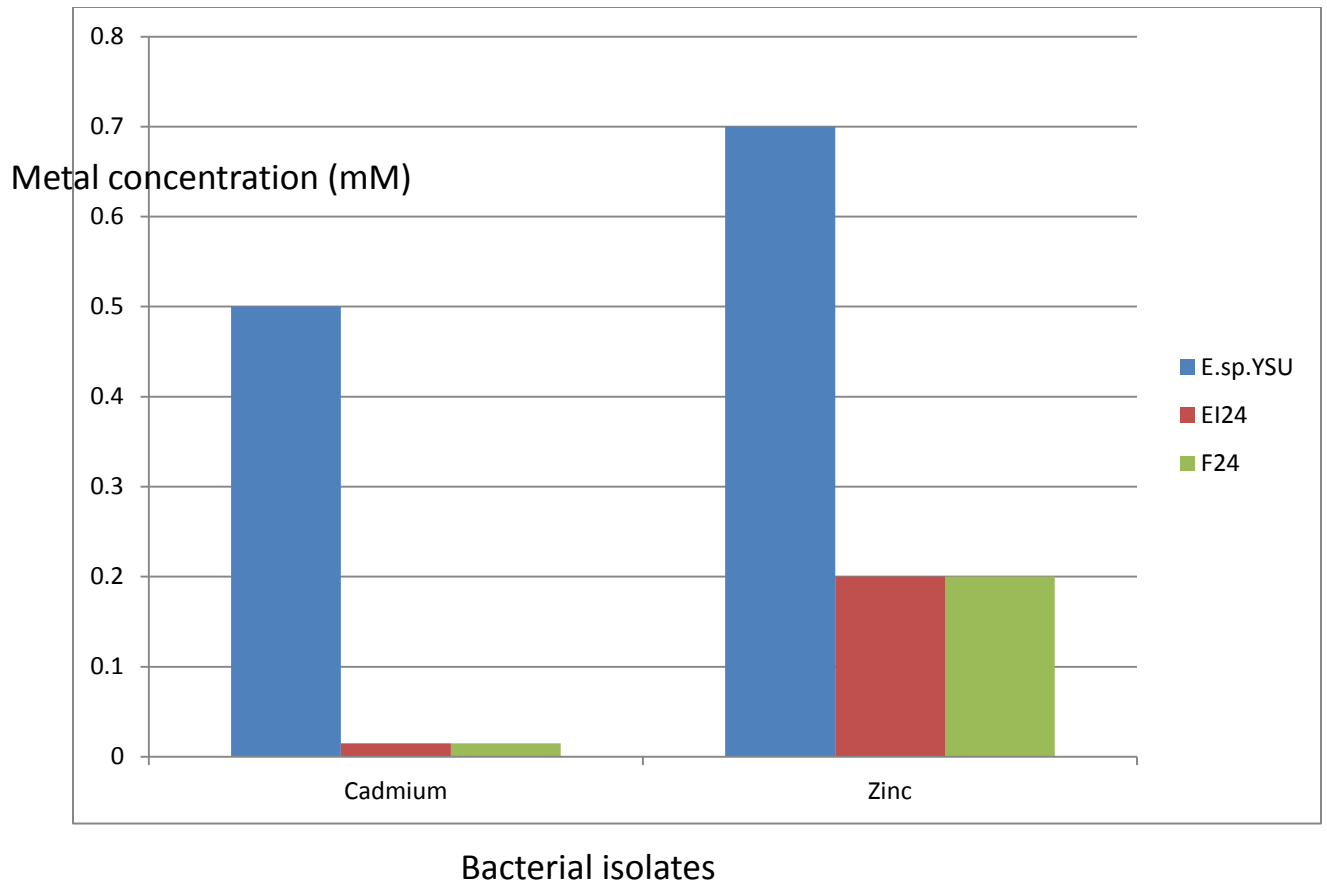


Fig. 2. The first set represents the MIC of *Enterobacter* sp.YSU and its mutants in cadmium while the second set of bars represents MICs in zinc. The blue bars represent the wildtype *Enterobacter* sp. YSU, the red bar represents EI24 mutant and the green bar represents the F24 mutant.

Replica plating was used to determine the phenotype of the mutants. This was achieved through comparison of the initial plates and the ensuing plates on which the colonies had been replica plated. Absence of colonies on the secondary plates that were present on the initial plates was an indication of the colonies being sensitive to some components of the media. The growth of the wildtype was then compared to the growth of the mutants in a media that usually supports the growth of *Enterobacter* sp. YSU.

In figures 1A and 1B, the plates have been replica plated with several mutants including EI24, F24 and the wildtype. Figure 1A is the cadmium plate at 0.4 mM. At this concentration the wildtype, indicated by the red box, grew while both mutants, indicated by the blue box, did not grow. In Figure 1B, the colonies were replica plated on an R3A-tris agar plate supplemented with 500 μ M of zinc. The wildtype, enclosed by the red box, had positive growth while the mutants, enclosed by the blue box, failed to grow. This established the phenotype of the mutants.

Gene rescue results

Genomic DNA was isolated from both mutants, partially digested with *BfuC* I and completely digested with *EcoR* I, *Sac* I, *Nco* I, *EcoR* V and *Aat* II (Fig.3). The digested DNA was self-ligated with T4 DNA ligase, concentrated by ethanol precipitation and transformed into ECD-100 *pir* cell by electroporation (80). Only those cells containing the transposon grew on LB-kan plates.

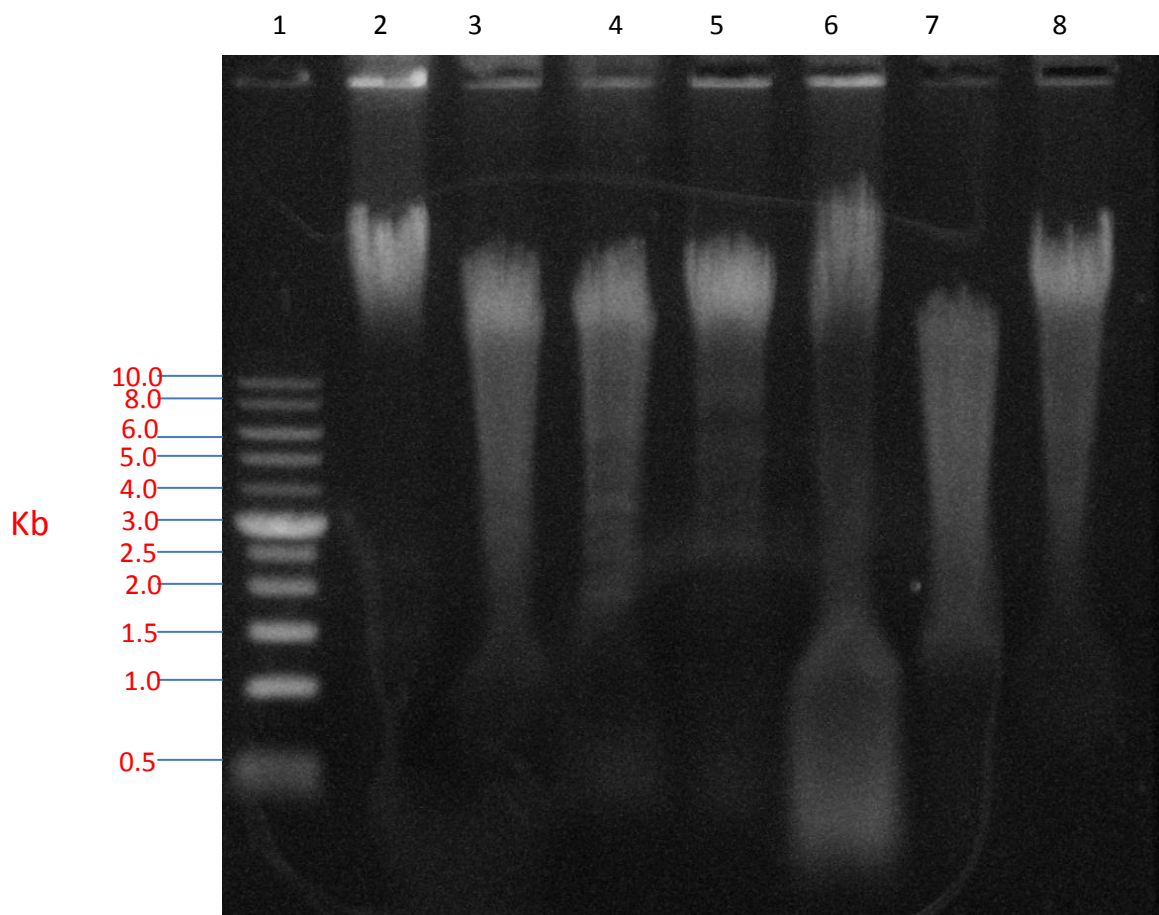


Fig. 3. Restriction digestion of EI24 genomic DNA mutant. Lane 1- Ladder, lane 2- undigested EI24, lane3- *Aat* II digested, lane 4- *Sac* I digested, lane 5- *EcoR* I digested, Lane 6- *BfuC* I digested, lane 7- *Nco* I digested, lane 8- *EcoR* V digested.

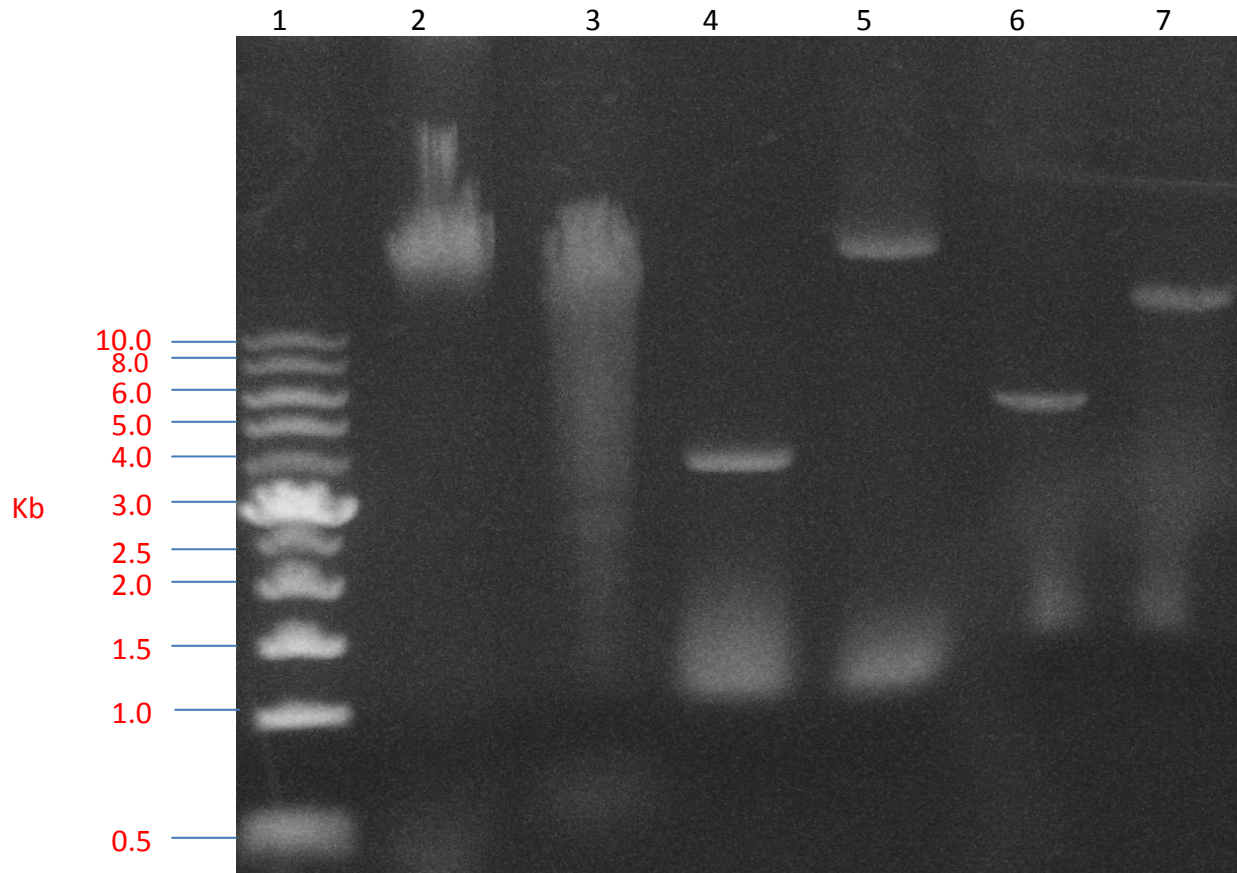


Fig 4. Lane 1- ladder, lane 2- F24 genomic DNA, lane 3- *Aat* II digested F24 DNA, lane 4- *Aat* II plasmid, lane 5- *Nco* I Plasmid, lane 6- *Aat* II plasmid linearized with *Xho* I, lane 7- *Nco* I plasmid linearized with *Xho* I

Electroporation transformation of the ligation mixture into ECD100 *pir* generated 2 transformants for EI24 mutant digested with *Sac* I, 65 transformants for EI24 mutant digested with *Nco* I, 50 transformants for *Aat* II digests, 103 for *EcoR* I digests, 20 for *BfuC* I digests, and 11 for *EcoR* V digests. The new plasmids were then purified (Fig. 4) and linearized with *Xho* I to obtain the actual size of the plasmids since it is not possible to obtain this information from the circular plasmids due to supercoiling.

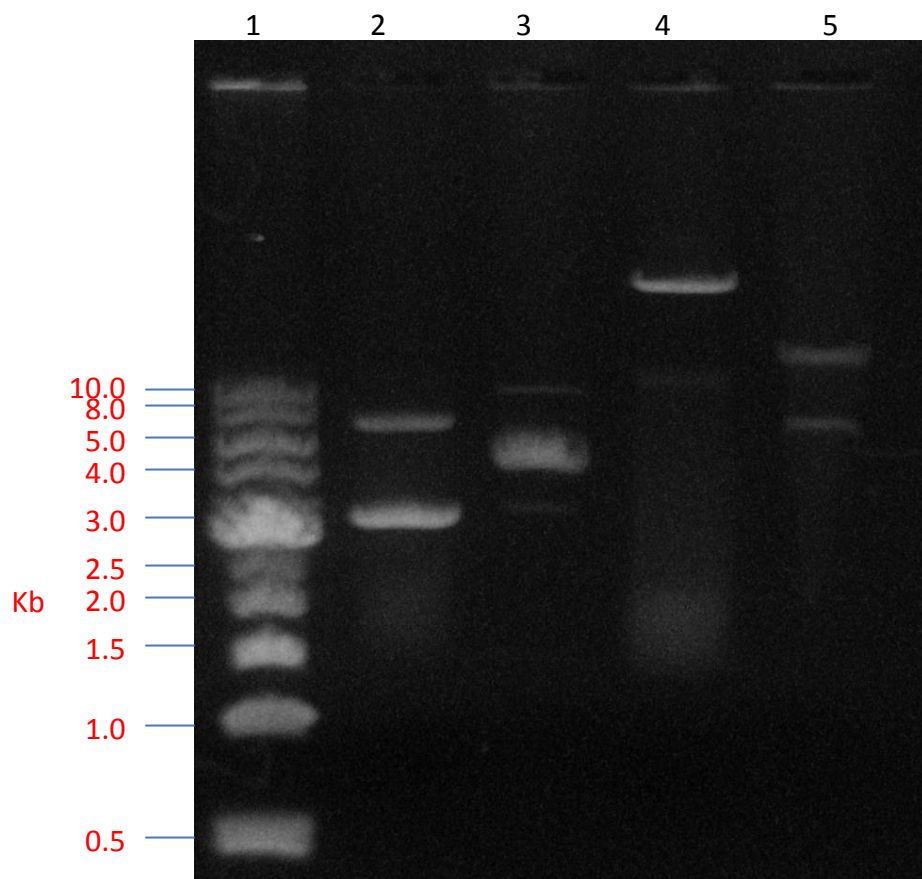


Fig. 5. *Xho* I linearized EI24 plasmids. Lane 1- 10 kb ladder, lane 2- *Nco* I plasmids, lane 3- *Nco* I Linearized with *Xho* I, lane 4- *Sac* I plasmid, lane 5- *Sac* I plasmid linearized with *Xho* I

Table 2 shows the plasmids used in sequencing and their various sizes. Undigested and digested plasmid samples were run on a gel. The difference in their migration pattern was an indication that the plasmids were successfully linearized. The two bands visible in some of the undigested samples were due to supercoiling of plasmid DNA. In the digested sample, if there is more than one band, they are added up and the sum is the actual size of the plasmid. The EI24 plasmid generated from *Sac* I had a band above the 10 Kb mark which was estimated to be about 13 Kb and another at the 7 Kb mark making it 20 Kb (Lane 5, fig.5). The linearized plasmids from F24 mutant had single bands.

Table 2. Linearized plasmid sizes used in sequencing

Plasmid	Size
EI24 <i>Nco</i> I generated plasmid	18 kb
EI24 <i>Sac</i> I generated plasmid	20 kb
F24 <i>Aat</i> II generated plasmid	6 kb
F24 <i>Nco</i> I generated plasmid	13 kb

Sequencing results

The KAN-2 FP-1 and R6KAN-2 RP-1 primers specific for the transposome DNA sequence, were used to obtain partial sequences that were related to a *Enterobacter cloacae* P-type ATPase involved in Zn (II), Cd (II), Pb (II), Hg (II) and Co (II) efflux as seen from the BLAST results (Fig.6). The map of the region from *Enterobacter cloacae* draft genome that contained the ATPase was downloaded and used as a guide in determining the rest of the sequence (Fig.9). Gene walking, a technique used in molecular biology to identify unknown sequences flanking a known DNA sequencing, was used to generate several sequences. The DNA fragments were then assembled to generate the contig sequence (Fig. 7) using VectorNTI. After several sequencing reactions, a TRAP protein was hit. Therefore, primers were designed to go through the TRAP protein and get the sequence after that towards the 5' end. From the composite EI24-F24 map, it is apparent that there is an overlap between the two mutants.

The assembled P-type ATPase sequence from *Enterobacter* sp. YSU was aligned with the P-type ATPases from *E. cloacae* and *E.coli* using MEGA5 (85). The protein multiple sequence alignment shows that a short sequence of about 80 amino acids at the 5' end is yet to be obtained (Fig. 10). At the 3' end, Lys⁶⁹³ and Asp⁷¹⁴ are conserved in all three ATPases. Studies done by Okkeri et al revealed that in *E. coli* ZntA, these residues are involved in binding of the translocated Zn²⁺ and Asp 714 is most especially important for the bioenergetic linkage of the metal binding site to the ATPase activity of ZntA (82).

Fig. 6. Basic Local Alignment Search Tool (BLAST) analysis of the putative P-type ATPase sequence from *Enterobacter* sp YSU.

Figure 7 is a Vector NTI diagram of the current sequence along with the location of the primers used in the sequencing reactions. Each reaction generated sequences that were approximately 200-500 base pairs long, and all of them were assembled into one long sequence using the ContigExpress software. The Zn F24 F2, Zn F24 F4, Zn F24 R5 and Zn F24 R6 primers yielded poor sequencing results. This was possibly caused by secondary structure in the DNA template downstream of the primer binding site. The transposome inserted between base pairs 130 and 131 to create the F24 mutant and between base pairs 155 and 156 to create the EI24 mutant.

EI24-F24 Composite - 5-21-12

	<u>Zn F24 R3 100.0%</u>									
1	CCACCGGGCA	TTTTTACCGA	CGGCGCAACA	GCGCGCAACG	GTTGCGCGTA	ACCAGCACCG	TGCGCCCGGT	ATCGCCAGC	ACGCCAGCC	ACAGGCCGGT
	GGTGCCCGGT	AAAAATGSGT	GCCGCGTTGT	CGCGGTTGCG	CAAGCGGCAT	TGGTCGTGGC	AGCGGGGGCA	TAGCGGCTCG	TGGCGGTGCG	TGTCGGGCA
	<u>Zn F24 F2 100.0%</u>									
101	CATGCCGAGC	AGCGTGGTGA	CGAGGAATAT	CCCTTCAGC	CCAGCGCAA	TCOCAATGTT	CTGGCGGATG	TGGCGCGCG	TGCGCCGCGC	CAGGCTAATC
	GTACGGCTCG	TGCACCACT	GCTCCTTATA	GGGGAAGTCG	GGTGCGGT	AGGGTTACAA	GACCGCTAC	AACCGCGCGC	AGCGGGCGCG	GTCGGATTAG
	<u>Zn F24 F4 100.0%</u>					<u>Zn F24 F1 100.0%</u>				
201	ATCTGCGCCA	GCCCGGTGAG	GCGGTTGTGG	GTCAGCGCGG	CATCCGCGGT	CTCCAGCGCC	ACATCGGTGC	CGCTGCCCAT	CGCAATGCGG	ATGGTGGAAAG
	TAGACGGGTT	CGGGCCAGTC	CGCCAACACC	CAGTGCGGCG	GTAGGCGGCA	GAGTCCGGG	TGTAGCCACG	GCGACGGGTA	CGGTAGCGGC	TACCACCTTC
	<u>Zn EI24 R1 100.0%</u>									
301	CCCTCATTCG	CGGGCATCG	TTTATCCCGT	CCCGACCAT	CGCCAGCGGG	GCATGACCGT	TCAGCTCGGT	TACCGCGCTG	ACTTTATCCG	CAGCAACAA
	GGAAAGTAACG	GCOCCTGAGC	AAATAGGGCA	GGGGCTGGTA	CGGGTCGCC	CGTACTGCGA	AGTCGAGCCA	ATGGCGCGAC	TGAAATAGGC	GTCGGTTGTT
	<u>Zn EI24 R1 100.0%</u>									
401	TCGGCCCTTA	AACTCCAGCC	CCAGCTCGCC	GGCAATCGCC	CGCCCGCGAC	GCGGGTTATC	ACCGGTACAG	ATCACGCCCT	GTACCCCGAG	CCTGTGCAGC
	AGGCCCGAAT	TTGAGGTCGG	GTCGAGCGG	CGCTTAGCGG	CGCGCGCGTG	CGCCAATAG	TGGCCAGTCC	TAGTCCGGGA	CATGGGGGTC	GGACACGTGC
	<u>HincII</u>									
501	CGTCTTTCGG	CGTCTTTCGG	ATCGTCAACG	AGGGTGTCCG	GTCAGCGCCG	CATGCCCATC	GGTACACCGT	CCTGCATCAC	GGCAACGACC	GTCTGCCCGG
	CGCAGCTGCC	GCAGAAAGCG	TAGCAGTCCG	TCCACACGCG	CGTCCGGTTC	GTACGGGTAG	CCATGTGGCA	GGACGTAGTG	CCGTTGCTGG	CAGACGGGGC
	<u>Zn F24 R1 100.0%</u>									
601	CGCTGCTTAA	TGCTCAACC	TGTGGATTG	GGAACGCGCC	TGCGCGGGG	ATCAGCACTT	TTTTGCCTTC	GAACACAGCC	TCAATCCCTG	ACCCGACCAG
	GGACGAGATT	ACGGAGTTGG	ACACCTAAGC	CCTTCCGCGG	ACGGCGCGCG	TAGTCGTGAA	AAAACGGAAG	CTGGTCTCGG	AGTTAGGGAC	TGGCTGTGTC
701	CGCTCGCTGG	GCGGTTGCGG	GAGGATGGT	CAGCCCGCGC	CCCTTCGCTT	CAGGCACAA	CGCCTCGCGC	AGAGGGTGGG	TGGAGCCCTG	CTCACGGCA
	GCGAGCGACC	CGCCAACGGC	CTCCCTACCA	GTCGGCGCGG	GGGAAGCGAA	GTGCGTGTTA	GCGGACGCGG	TCTCCACCC	ACCTCGGGAC	GAGTGCCTGT
801	GCGCCAGCGG	TCAGCAGGGC	GTCTTCACTG	ATGTCCTGGG	GATACAGCGC	GGTCACTGCG	GGCTTGCGGA	CGGTGAGCGT	GCGGTTTTTA	TGCAAGGGCA
	CGCCGCTCCG	AGTCGTCCCG	CAGAAGTGAC	TACAGGAGCG	CTATGTGCGG	CCAGTGGAGC	CGAAGCGGCT	GCCAGTCCGA	CGGCCAAAAT	AGCTTCCGCT
901	TGTGCTGAAC	CTGGCTCAGC	TGTTCCAGCG	CGCGCCCGCC	CTTAATCAGC	GCOCGCGCAC	GTGCGCGCGC	CGCAGCCCTT	GAGGTAATCG	CGCCGCGGGT
	ACACGACTTG	GACCGAGTCC	ACAAGGTGCG	GGCGCGCGCG	GAATTAGTCC	CACGGCGCGC	GCGGTCGGGA	CTCCATTAGC	GCGCGCCCCA	
1001	GGAGATCAC	AGCGCACAGC	GACAGCCGAT	CAGCAGCAGC	GTCAGCCCTT	TGTAATTCOA	GCCCTCCCAC	GGCGCGCGGA	AGAACAGCGG	CGGGACGAGC
	CCTCTAGTGG	TCGCGGTGTC	CTGTGCGCTA	GTCGTCGTCC	CAGTCCGGAA	ACATTAGGTT	CGGGAGGGTG	CCGCGCGGCT	TCTTGTGCGC	GCCTGCTGTC
	<u>Zn F24 F3 100.0%</u>									
	<u>Zn F24 R2 100.0%</u>									
1101	GTGACCAGCA	GCGCCACCAG	CATGATGGCG	GGGGTGTAAT	TCCGGCTGAA	GCGATCGATA	AAGCGTTCCTA	CCGGCGCGCG	GCGCTCCTCG	GCTTCTTCTA
	CACTGGTGGT	CGCGGTGGTC	GTACTACGCG	CCCCACATTT	AGGCCGACTT	CGTAGCTAT	TTCGCAAGAT	GGCGCGCGCG	CGCGAGGAGC	CGAAGAAGAT
	<u>Zn F24 R2 100.0%</u>					<u>HincII</u>				
1201	TCAGCTGAG	GATGCGGTCG	ATGGCGCTGT	CGCCCGGTTG	GAAAAGGACC	GTCAGCAGCA	CCAGTCCGTC	GAGGCTGGTG	GCGCCCGCAG	GCACCTTTTC
	AGTCGGACTC	CTACGCCAGC	TACCGGACA	GCGGCGCAAG	CCTTTCTCGG	CAGTCTGCTT	GGTCAGCCAG	CTGCGACCAC	CGCGGGCGTC	CGTGAAAGG
	<u>AatII</u>									
1301	ACCTGCGCGG	GCGCTACCG	GGATGGATTG	CCCGGTCAGG	GCGCTTTCGT	CAAAGCTCGC	GGTGGCGGTA	ATGAGGGCC	CATCGGCTGG	CAGACGTCCG
	TGGACGGCGC	GCGGATGGC	CCTACCTAAG	GGCCAGTCC	CGCGAAAGCA	GTTCGAGCG	CCACCGCCAT	TACTCCCGGG	GTAGCCGACC	GTCTGACAGG
1401	CCTGCGGGCA	CTTCAATCAC	GTCGCCCGGG	CGCAGGGTAT	TGATGGCGAC	CGTTTACCGC	TGACCAGGAA	TAACCCCGGT	GCGGTTTTTC	GGCTTACAGG
	GGACGCCCGT	GAAGTTAGTG	CAGCGGGCCC	GCGTCCCATA	ACTACCGCTG	GCAAAGTGCG	ACTGGTCTT	ATTGGGCGCA	CGGCCAAAGG	CCGAAGTCCG
	<u>Zn F24 F5 100.0%</u>									
1501	CCATCAGCGC	GCTCACCCCT	TTACGCGCCC	GGCTGCGCGG	CCAGCCCTCA	AGGCGTTCCG	CAATTAAGAA	CAGCAGCAGC	ACAATCGCGG	CTTCCGGCGT
	GGTAGTCGCG	CGAGTGGGGA	AAATGCGCGG	CCGAGCGCGG	GGTCCGGAGT	TCCGCAAGCG	GTAAATCTCT	GTCGTCGCTG	TGTTAGCGCG	GAAGCCGGCA
1601	GCGCCAAATA	AACAGCGCCC	CGATAGCCCG	CACGCTCATC	AGCGTTTCGA	TGGCAAACCA	GCTGCCGCTT	CTCATCAGAC	GCAAGCCCTG	ACCGCAATA
	GCGCGGTTAT	TTGTGCGGGG	GCTATCGGCG	GTGCGAGTAG	TCGCAAAGCT	ACGTTTGGT	CGAGCGCGAA	GAGTAGTCTG	CGTCCGCGAC	TGCGGTTTAT
	<u>Zn F24 R4 100.0%</u>					<u>Zn F24 R5 100.0%</u>				
1701	GGGAACAGCG	CAACCAGCGT	GGTGGCGATA	AACGCCAGGT	TGCCGAACGG	GTGGTTGATT	TGCTCCAGCC	CCCAGCTCAG	GGCCATCATG	ATGATGAGGG
	CCCTGTGCCG	GTTGTCGCA	CCACCGCTAT	TTGCGGTCCA	ACGGCTTGCC	CACCAACTAA	ACGAGGTCGG	GGGTGAGTCC	CCGGTAGTAC	TACTACTCCC
	<u>Zn F24 F6 100.0%</u>									
	<u>Zn F24 R6 100.0%</u>									
1801	TAATGAGCGG	CAGATTTTAT	TTTAGCGGGG	ACGTTTTTTC	TGCCGCGCGC	GTTCGCTGCG	GCAGGGTATA	ACCCGCTTTG	CTGACGGCGC	CATCAACCTG
	ATTACTCGCG	GTCTAAAATA	AAATCGCCCC	TGCAAAAAAG	ACGCCCGCGG	CAAAGCGAGC	CGTCCCATAT	TGGGGAAAC	GACTCGCGCG	GTAGTTGGAC
1901	TCTGCTGAGC	TATTTTACCG	CCCCGACCAC	CAGCTTTTTC	GTAACAACCA	GCACCTGA				
	AGACGACTGC	ATAAAAATGGC	GGGCTGGTGG	GTCGAAAAGC	CATTGTTTTG	CGTGGACT				

Fig. 7 EI24-F24 the assembled sequence. The words in blue are restriction sites and the ones in red are various primers designed during sequencing. Their positions represent the position in the sequence where the sequence would begin

The DNA coding sequence (Accession FP929040) that matched the BLAST result in figure 6 was downloaded and aligned with the reverse complement of the YSU sequence as shown in figure 8. According to this diagram, approximately 225 base pairs are missing from the 5' end of the YSU ATPase gene. The 3' end of the gene is intact.

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*      20      *      40      *      60      *      80      *      100
FP929040-A : ATGTGGACTCCAGAAATACCTAAAAAGTTCCCAATTCTCGGCACTTAAGCTCAGCCCGGTTCCGGCCAAAAGAGGACTGCTGCTGGGAGGGGGGGTGGG : 100
YSU-ATPase : ----- : -

*      120     *      140     *      160     *      180     *      200
FP929040-A : AAAACCCGACCCGAAGCGCTGCCTGAAAACCGGCMACTCACTACAGCTGGGTGGTCAACGGCATGGACTGGCGGGCTGGGGCCDCAAAAGTGGAAAACGGGT : 200
YSU-ATPase : ----- : -

*      220     *      240     *      260     *      280     *      300
FP929040-A : GAAGCAGGTGCCCCGTGTGAACCCGCTTCAGCTCTCTTTCCTACCGAAAAGCTCTGGTCAACCCGCAAAACACCTCAGCAGCAGCTTGAACCCGCG : 300
YSU-ATPase : ----- : 73
TCAGGTCTCTTTCCTACCGAAAAGCTCTGGTCAACCCGCAAAACACCTCAGCAGCAGCTTGAACCCGCG
TCAGGTCTCTTTCCTACCGAAAAGCTG TGGTC G GC G AAA ACCTCAGCAGCAGCTTGA CCGCGC

*      320     *      340     *      360     *      380     *      400
FP929040-A : CTCAGCAAGCCGGTTATACCTTCCGCGGCAATCGCCCGCCCGCAGAAAACCTGCTCCGCTCAAAACAAACCTGCCCTATACCCCTCATCATCATG : 399
YSU-ATPase : CTCAGCAAGCCGGTTATACCTTCCGCGGCAATCGCCCGCCCGCAGAAAACCTGCTCCGCTCAAAACAAACCTGCCCTATACCCCTCATCATCATG : 173
CTCAGCAAGCCGGTTATACCTTCCGCGGCAATCGCCCGCCCGCAGAAAACCTGCTCCGCTCAAAACAAACCTGCCCTATACCCCTCATCATCATG

*      420     *      440     *      460     *      480     *      500
FP929040-A : ATGCCCCCTCAGCTGGGGCTGGAGCAATCAACCCCGCGTCGGCAACCTGGGCTTTATCGCCACCACCGCTGGTGGCCCTCTCCCAATTGGCCCTCAGG : 499
YSU-ATPase : ATGCCCCCTCAGCTGGGGCTGGAGCAATCAACCCCGCGTCGGCAACCTGGGCTTTATCGCCACCACCGCTGGTGGCCCTCTCCCAATTGGCCCTCAGG : 273
ATGCCCCCTCAGCTGGGGCTGGAGCAATCAACCCCGCGTCGGCAACCTGGGCTTTATCGCCACCACCGCTGGTGGCCCTCTCCCAATTGGCCCTCAGG

*      520     *      540     *      560     *      580     *      600
FP929040-A : CCGTCGCTCTCATGCAAGCCCGCAGCTCGTTTGCATCGAAAAGCTCATGAGCTGGGGCTATCGGGCCCTCTTTATTGGCCGACCGCCGAAGCCGG : 599
YSU-ATPase : CCGTCGCTCTCATGCAAGCCCGCAGCTCGTTTGCATCGAAAAGCTCATGAGCTGGGGCTATCGGGCCCTCTTTATTGGCCGACCGCCGAAGCCGG : 373
CCGTCGCTCTCATGCAAGCCCGCAGCTCGTTTGCATCGAAAAGCTCATGAGCTGGGGCTATCGGGCCCTCTTTATTGGCCGACCGCCGAAGCCGG

*      620     *      640     *      660     *      680     *      700
FP929040-A : GATCTGCTGCTGCTCTTCTTAATGGCGAAGCGCTTTCAGGGCTGGGGCCCGCGCCCGCCGCTAAAGGGGTGAGCCGCTCATGGCCCTGAAGCCGGAA : 699
YSU-ATPase : GATCTGCTGCTGCTCTTCTTAATGGCGAAGCGCTTTCAGGGCTGGGGCCCGCGCCCGCCGCTAAAGGGGTGAGCCGCTCATGGCCCTGAAGCCGGAA : 473
GATCTGCTGCTGCTCTTCTTAATGGCGAAGCGCTTTCAGGGCTGGGGCCCGCGCCCGCCGCTAAAGGGGTGAGCCGCTCATGGCCCTGAAGCCGGAA

*      720     *      740     *      760     *      780     *      800
FP929040-A : ACCGCCAGCCGGTTATTCCTGCTCAGCTGAACCGCTCGCCATCAATACCTTGGCCCGCCGACCTGATTGAAGTCCCGCCAGCGGAGCTCTGCCAG : 799
YSU-ATPase : ACCGCCAGCCGGTTATTCCTGCTCAGCTGAACCGCTCGCCATCAATACCTTGGCCCGCCGACCTGATTGAAGTCCCGCCAGCGGAGCTCTGCCAG : 573
ACCGCCAGCCGGTTATTCCTGCTCAGCTGAACCGCTCGCCATCAATACCTTGGCCCGCCGACCTGATTGAAGTCCCGCCAGCGGAGCTCTGCCAG

*      820     *      840     *      860     *      880     *      900
FP929040-A : CGCATGGGGCCCTCMTACGCCACCGCGAGCTTTCAGCAAGGCGCCCTGACCGGGGAATCCATCCCGCTACCGCCCGCCGAGCTCAAGCTGCTCTCG : 899
YSU-ATPase : CGCATGGGGCCCTCMTACGCCACCGCGAGCTTTCAGCAAGGCGCCCTGACCGGGGAATCCATCCCGCTACCGCCCGCCGAGCTCAAGCTGCTCTCG : 673

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CCGATGGGGCCCTCATTAACGCCACCGGAGCTTTGACGAAAGCGCCCTGACCGGGAATCCAATCCGGTAGCGCGCGCGCAGGTGA AAGGTGCCTGC

* 920 * 940 * 960 * 980 * 1000

FP929040-A : GGGCGCCAACAGCGT CGA CCGGACTGGTGTCTGCTGA CCGTCCCTTCGAAACCGGG - CGA CAGCGCCATCGA CCGCATCCTCA GGCCTGATGAA GAGAGCGCG : 998
 YSU-ATPa.se : GGGCGCCAACAGCGT CGA CCGGACTGGTGTCTGCTGA CCGTCCCTTCGAAACCGGG CGA CAGCGCCATCGA CCGCATCCTCA GGCCTGATGAA GAGAGAGCGCG : 773
 GGGCGCCAACAGCGT CGA CCGGACTGGTGTCTGCTGA CCGTCCCTTCGAAACCGGG CGA CAGCGCCATCGA CCGCATCCTCA GGCCTGAT GAA GA GC CGA

* 1020 * 1040 * 1060 * 1080 * 1100

FP929040-A : GGA GCGCCGCGCGCCGT GGA GCGGTTGAT CGA CCGCTTCAGCCGAGTTTACA CCCC GCCATCATGCTGGTGGCCTGCTGGT CACCGTGGTCCGCGCG : 1098
 YSU-ATPa.se : GGA GCGCCGCGCGCCGT GGA GCGGTTGAT CGA CCGCTTCAGCCGAGTTTACA CCCC GCCATCATGCTGGTGGCCTGCTGGT CACCGTGGTCCGCGCG : 873
 GGA GCGCCGCGCGCC GT GA CG TT ATCGA CCGCTTCAGCCGAGTTTACA CCCC GCCATCATGCTGGT GC CTGCTGGT CACCGTGGTCCGCGCG

* 1120 * 1140 * 1160 * 1180 * 1200

FP929040-A : CTGTTCTTCGCGCGCCGTTGGAGGGCTGGATTTA CAAAGGCTGACGCTGCTGCTGATCGGCTGTCCTGTGCGCTGGT GATCTCCACCCCGCGCG : 1198
 YSU-ATPa.se : CTGTTCTTCGCGCGCCGTTGGAGGGCTGGATTTA CAAAGGCTGACGCTGCTGCTGATCGGCTGTCCTGTGCGCTGGT GATCTCCACCCCGCGCG : 973
 CTGTTCTTCGCGCGCCGTTGGAGGGCTGGATTTA CAAAGGCTGACGCTGCTGCTGATCGGCTGTCCTGTGCGCTGGT GATCTCCACCCCGCGCGA

* 1220 * 1240 * 1260 * 1280 * 1300

FP929040-A : TTA CCTCAGGCTGGCGCGCGGCAGCGCGCGGCGTGATTAAGGGCGGCGCGGCTGGAG CAGCTGAGCCAGTTT CAGCACATCGCCTT CGATAA : 1298
 YSU-ATPa.se : TTA CCTCAGGCTGGCGCGCGGCAGCGCGCGGCGTGATTAAGGGCGGCGCGGCTGGAG CAGCTGAGCCAGTTT CAGCACATCGCCTT CGATAA : 1073
 TTA CCTCAGGCTGGCGCGCGGCAGCGCGCGGCGTGATTAAGGGCGGCGCGGCTGGAG CAGCTGAGCCAG TT CAGCACATCGCCTT CGATAA

* 1320 * 1340 * 1360 * 1380 * 1400

FP929040-A : AACCGGCAAGCTGACCGT CGGCAAGCCGAGGTGA CCGCGTGTA TCCCGAGGACATCAGTGAAGAGCGGCTGCTA CTGGCCGCTGCGGT GAGCAG : 1398
 YSU-ATPa.se : AACCGGCAAGCTGACCGT CGGCAAGCCGAGGTGA CCGCGTGTA TCCCGAGGACATCAGTGAAGAGCGGCTGCTA CTGGCCGCTGCGGT GAGCAG : 1173
 AACCGGCAAGCTGACCGT CGGCAAGCCGAGGTGA CCGCGTGTA TCCCGAGGACATCAGTGAAGAGCG TGCT A CTGGCCGCTGCGGT GA CAG

* 1420 * 1440 * 1460 * 1480 * 1500

FP929040-A : GGCCTCACCCACCCTCTGCGGCAAGCGATTGTGCGTGAGCGAAGGGCGCGGCTGACCATCCCTCCGGCAACGCCCA GCGAGCGCTGGT CGGGT CAG : 1498
 YSU-ATPa.se : GGCCTCACCCACCCTCTGCGGCAAGCGATTGTGCGTGAGCGAAGGGCGCGGCTGACCATCCCTCCGGCAACGCCCA GCGAGCGCTGGT CGGGT CAG : 1273
 GGCCTCACCCACCCTCTGCGGCAAGCGATTGTGCGTGAGCGAAGGGCGCGGCTGACCATCCCTCCGGCAACGCCCA GCGAGCGCTGGT CGGGT CAG

* 1520 * 1540 * 1560 * 1580 * 1600

FP929040-A : GGA TTGAGGCTGTGGTCGAAAGCAAAAAGTGATGATCGCGCGCAGGCGGCTTCCGAAATCCACAGGTGAGGCATTAGAGCAGGCGGGCAGAGGTT : 1598
 YSU-ATPa.se : GGA TTGAGGCTGTGGTCGAAAGCAAAAAGTGATGATCGCGCGCAGGCGGCTTCCGAAATCCACAGGTGAGGCATTAGAGCAGGCGGGCAGAGGTT : 1373
 GGA TTGAGGCTGTGGTCGAAAGCAAAAAGTGATGATCGCGCGCAGGCGGCTTCCGAAATCCACAGGTGAGGCATTAGAGCAGGCGGGCAGAGGTT

* 1620 * 1640 * 1660 * 1680 * 1700

FP929040-A : TGTGCGGTGAGCAGGACGGTGTACCGATGGGCA TGTGCGCTGCGCGACA CCGTGGTGA CCGATGCGAAAAGCAGCGT CGA CCGCTGCACAGGCTG : 1698
 YSU-ATPa.se : TGTGCGGTGAGCAGGACGGTGTACCGATGGGCA TGTGCGCTGCGCGACA CCGTGGTGA CCGATGCGAAAAGCAGCGT CGA CCGCTGCACAGGCTG : 1473
 TGTGCGGTGAGCAGGACGGTGTACCGATGGGCA TGTGCGCTGCGCGACA CCGTGGTGA CCGATGCGAAAAGCAGCGT CGA CCGCTGCACAGGCTG

* 1720 * 1740 * 1760 * 1780 * 1800

FP929040-A : GGGGTACAGGCGGTATCTGACCGGTGAT AACCCGCGTGGCGCGCGCGGATGCGCGGAGCTGGGCTGGAGTTTAAAGGCCGATTGTTGCTGCGG : 1798

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YSU-ATPase : GGGGTACA GGGCGTGATCCTGACCGGTGAT AACCCGCTGCGGCGGCGCGAT TGCCGCGAGCTGGGCTGGAGTTTAAAGGCCGATTGTTGCCTGCGG : 1573
GGGGTACA GGGCGTGATCCTGACCGGTGAT AACCCGCTGCGGCGGCGCGAT TGCCGCGAGCTGGGCTGGAGTTTAAAGGCCGATTGTTGCCTGCGG

          *      1820      *      1840      *      1860      *      1880      *      1900
FP929040-A : ATAAAGTCAGCGCGGTAAACCGAGCTGAAACGGTCATGCCCGCTGGCGATGGTCGGGACGGGATAAACGATGCCCGGCAATGAAGGCTTCCACCATCGG : 1898
YSU-ATPase : ATAAAGTCAGCGCGGTAAACCGAGCTGAAACGGTCATGCCCGCTGGCGATGGTCGGGACGGGATAAACGATGCCCGGCAATGAAGGCTTCCACCATCGG : 1673
ATAAAGTCAGCGCGGTAAACCGAGCTGAAACGGTCATGCCCGCTGGCGATGGTCGGGACGGGATAAACGATGCCCGGCAATGAAGGCTTCCACCATCGG

          *      1920      *      1940      *      1960      *      1980      *      2000
FP929040-A : CATTCGATGGGCAGCGGCAACGATGTGGCCCTGGAGACGGCGGATGCCGGCGTGACCCAAACCCGCTGACCGGCTGGCCAGATGATTAACCTGGCCG : 1998
YSU-ATPase : CATTCGATGGGCAGCGGCAACGATGTGGCCCTGGAGACGGCGGATGCCGGCGTGACCCAAACCCGCTGACCGGCTGGCCAGATGATTAACCTGGCCG : 1773
CATTCGATGGGCAGCGGCAACGATGTGGCCCTGGAGACGGCGGATGCCGGCGTGACCCAAACCCGCTGACCGGCTGGCCAGATGATTAACCTGGCCG

          *      2020      *      2040      *      2060      *      2080      *      2100
FP929040-A : CCGGCACGCGCGCCAACATCCGCCAGAACATTGGGATTCGCTGGGCTGAAAGGGATAATTCCTCGTCAACGCTGCTCGGCATGACCGGCTGTGGC : 2098
YSU-ATPase : CCGGCACGCGCGCCAACATCCGCCAGAACATTGGGATTCGCTGGGCTGAAAGGGATAATTCCTCGTCAACGCTGCTCGGCATGACCGGCTGTGGC : 1873
CCGGCAGCGCGCCAACATCCGCCAGAACATTGGGATTCGCTGGGCTGAAAGGGATAATTCCTCGTCAACGCTGCTCGGCATGACCGGCTGTGGC

          *      2120      *      2140      *      2160      *      2180
FP929040-A : TGGCGGTA CTGGCGGATACGGGGCGACGGTCTGTGTTACGGCGAACGCGTTGCGGCTGTTCGCGCTCGGTAAA----- : 2173
YSU-ATPase : TGGCGGTA CTGGCGGATACGGGGCGACGGTCTGTGTTACGGCGAACGCGTTGCGGCTGTTCGCGCTCGGTAAAATGCCCGGTTGG : 1960
TGGCGGTA CTGGCGGATACGGGGCGACGGTCTGTGTTACGGCGAACGCGTTGCGGCTGTTCGCGCTCGGTAAA

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Fig. 8. DNA Sequence alignment of the putative *Enterobacter* sp YSU P-type ATPase (bottom sequence) with *Enterobacter cloacae* Zn, Cd, Pb, Hg and Co P-type ATPase (FP929040 – Top sequence). An additional 225 bp on the 5' end are required to complete the *Enterobacter* sp YSU P-type ATPase sequence. Nucleotides shaded in black represent an identical match between the two sequences

A 13 kb region (FP929040) containing the *E. cloacae* P-type ATPase gene and the genes flanking it was imported into Vector NTI and mapped in Figure 9. The transposome plasmid generated by the *Aat* II digestion was used as a sequencing template along with the Zn F24 R2 primer (Fig. 7). BLAST analysis showed that the sequence downstream of the *Aat* II was almost identical to a region encoding a short peptide for a TRAP-type C4-dicarboxylate transport system. This result suggests that this region between *Enterobacter* sp. YSU and *Enterobacter cloacae* subspecies *cloacae* NCTC 9394 is conserved.

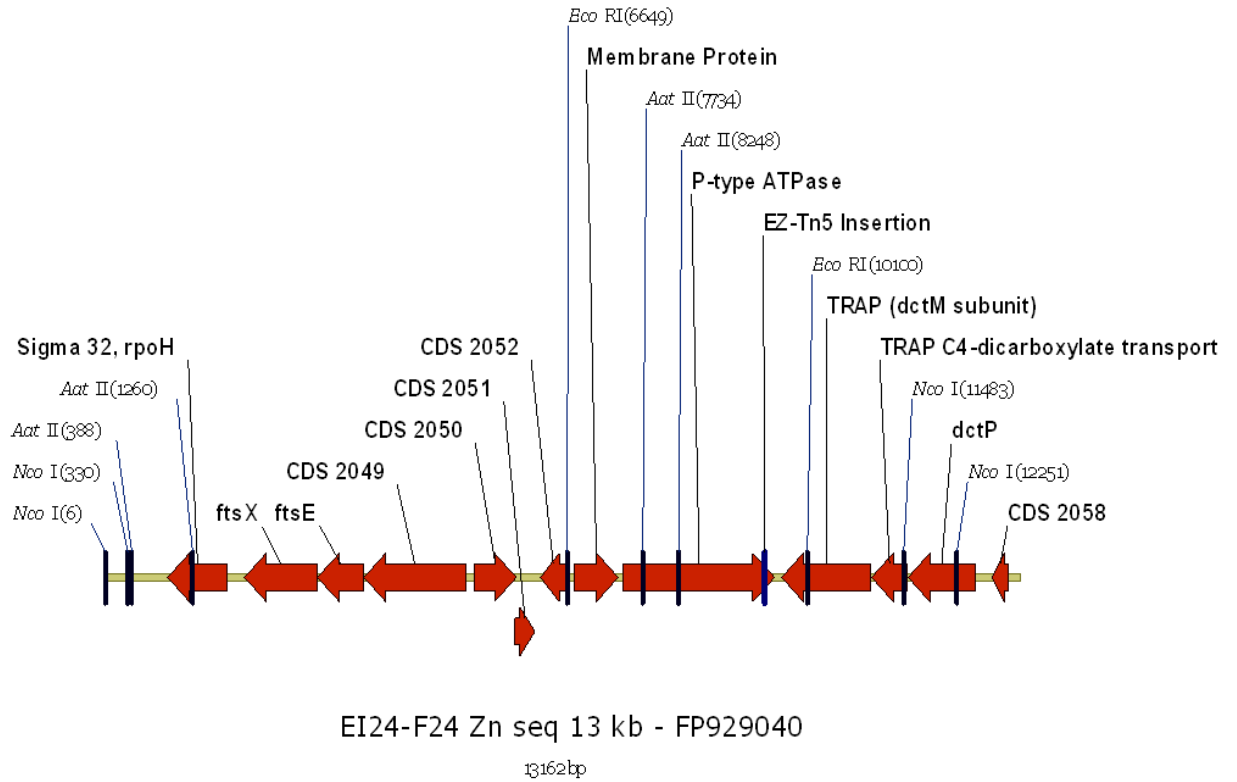


Fig.9 VectorNTI map of the identified P-type ATPase. The thin dark vertical line represents restriction sites. The thick red arrows represent gene sequences. The P-type ATPase is flanked by TRAP and Membrane protein on either side.

Figure 10 is an alignment of the amino acid residue sequence of the YSU P-type ATPase with the amino acid residue sequences of the *Enterobacter cloacae* P-type ATPase and the E. coli ZntA. This shows that DNA sequence for about 80 amino acid residues is required to obtain the complete sequence for the YSU P-type ATPase.

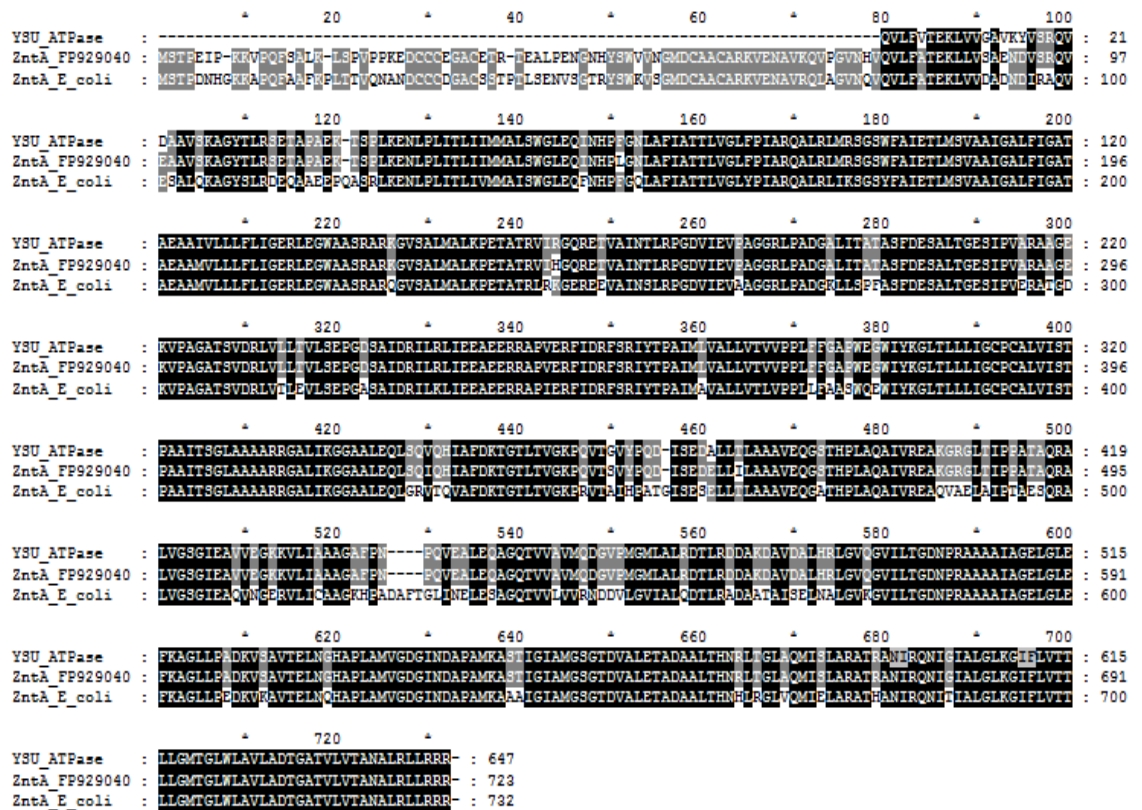


Fig. 10 Alignment of the translated *Enterobacter* sp. YSU P-type ATPase with Zn/Cd/Pb/Hg/Co translocating P-type ATPase (Accession # FP929040). The YSU ATPase amino acid residues, NI and IF, in black letters and shaded in grey represent the transposon insertion sites for EI24 between positions 682 and 683 and for F24 695 and 696.

CHAPTER IV

DISCUSSION

The results above support the hypothesis which predicted that zinc and cadmium resistance would be encoded by a P-type ATPase, RND or CDF efflux system to be true. The efflux system identified here was a Cpx-type ATPase which is a subgroup of P-type ATPases responsible for translocating toxic metals from the cytoplasm to the periplasm.

The F24 and EI24 mutants derived from *Enterobacter* sp. YSU seemed to be sensitive to zinc and cadmium. BLAST results revealed that the interrupted gene has a very high (95%) similarity to a P-type ATPase involved in transport of zinc, cadmium, mercury and lead in *Enterobacter cloacae* sbsp. However, since P-type ATPases export toxic metals from the cytoplasm to the periplasm, it is possible that an RND system may be involved in exporting the cations from the periplasm to the extra-cellular medium. This was suggested by Legatzki et al where the Czc efflux system, ZntA and CadA P-type ATPases of *Ralstonia metallidurans* were studied to see if there is interplay between them (69). In that same study it was also shown that there is interplay between CadA and ZntA P-type ATPases in *Ralstonia metallidurans* (69). ZntA and CadA may compensate for each other in the absence of one but neither of these P-type ATPases confers resistance to lead.

Mercury resistance

It was not possible to establish a phenotype for the mutants as well as the wildtype in mercury since the focus was mainly on zinc and cadmium. More MICs would have to be done to obtain this information.

Mercury resistance genes are arranged in the *mer* Operon consisting of *mer R*, *mer T*, *mer P* and *mer A*. *Mer A* is responsible for conversion of Hg(II) ions into the elementary form, Hg(0) (79). The fact that *Mer A* was not knocked out could be a probable cause for lack of sensitivity to mercury by the mutants. When *Mer A* is present, it actively reduces mercury to its non-toxic form but if it was absent, then perhaps the P-type ATPase would somehow compensate for that.

Lead resistance

There is not much known about bacterial resistance to lead compared to other metals. Some bacterial strains such as *Staphylococcus aureus*, *Citrobacter freundii* and *Pseudomonas marginalis* can survive in toxic concentrations of lead by precipitating it either as a phosphate salt or an extracellular polymer (70, 71). Of the few resistance determinants known to be specific to lead, one has been found in *Cupriavidis metallidurans* CH34. In a mutated strain in which *zntA* and *cadA* had been knocked out, expression of *pbrA* increased resistance to zinc 16-fold and to cadmium 128-fold.(72) In another mutant in which several important plasmid encoded metal resistance genes were knocked out but *zntA* and *cadA* were left intact, expression of *pbrA* had no effect on zinc and cadmium resistance (72). This was because when ZntA and CadA were present they efficiently removed the zinc and cadmium from the cytoplasm. However, in the *zntA*

/cadA⁻ mutant, *pbrA* compensated the loss for the loss of *ZntA* and *CadA* by taking over the export of zinc and cadmium (72).

The current study did not look at lead but the BLAST results show that the same p-type ATPase that is responsible for zinc and cadmium resistance also plays a role in lead resistance. Perhaps, in the same way that *ZntA* compensates for the loss of *CadA* and vice versa (69) *ZntA* may compensate for the absence of *pbrA*.

Re-cloning the identified genes into their original hosts is a way of confirming the function of the identified genes.

From the map generated by VectorNTI, it is clear that the EZ:Tn5 transposome inserted towards the 3' end. This end contains Lys⁶⁹³ and Asp⁷¹⁴ conserved residues responsible for binding of translocated zinc as shown in *ZntA* of *E. coli*. D⁷¹⁴ is particularly involved in linking of the metal binding site to the activity of the ATPases through bioenergetics (82). This could explain why the function of the ATPase was disrupted when the transposome inserted at that end. In a study of the metal-binding sites of the zinc-transporting P-type ATPase of *E. coli*, B. Mitra et al showed that the motif, GXXCXXC found on the N-terminal is not essential for efflux although it enhances the activity of the ATPase by regulating transport (81, 88). This suggests that there may be other sites that are essential for efflux. In plants, particularly *Thalapi caerulescens*, peptide segments cloned from the C-terminal of the P-type ATPase were found to be involved in cadmium tolerance (83). These studies suggest that the C-terminal end of Cpx-type ATPases may be involved in efflux of toxic cations from the cytoplasm.

TRAP protein

The Tripartite ATP-independent periplasmic protein (TRAP) that appears at the end of the contig sequence belongs to a family of transporters that are comprised of three components: (1) a small membrane protein that has 4 transmembrane segments, (2) a larger membrane protein that has 12 transmembrane segments, (3) a periplasmic substrate binding protein (74). These transporters utilize the substrate binding protein (SBP) to bind to the ligand and deliver the substrate to the membrane translocator for transport across the membrane. They do not utilize energy from ATP hydrolysis as is the case of P-type ATPases, but instead use energy stored in an electrochemical gradient to drive uphill substrate transport (75, 76). In the *Enterobacter cloacae* draft genome, this protein is located right next to the P-type ATPase. When the region next to the P-type ATPase was downloaded and subjected to BLAST analysis, it matched up to a TRAP protein. This region was rescued together with the P-type ATPase during the gene rescue process.

There are few substrates for the TRAP protein in bacteria that have been identified. In *E. coli* strain K-12, the identified substrate is 2, 3-diketo-L-gulonate (77) which is formed by the hydrolysis of dehydro-L-ascorbate, a spontaneous occurrence in aqueous environments which have neutral pH. However, this sole TRAP protein identified in *E. coli* has not been well studied because its substrate is unstable and even deletion of the genes encoding the transporter failed to establish a clear growth phenotype (78).

Conclusion

Bacteria have adaptations that enable them to survive in highly contaminated environments. Their ability to survive in environments contaminated with high concentrations of heavy metals has been correlated with their ability to develop antibiotic resistance. (67,68). *Enterobacter* sp. YSU, a highly resistant bacteria isolated from East Fork Poplar Creek exhibited high resistance to several heavy metals including zinc, cadmium, mercury and lead. The two mutants, F24 and EI24, were created by way of transposon mutagenesis to identify the gene responsible for these resistances. The disrupted sequence that was interrupted was found to be a P-type ATPase that is involved in zinc, cadmium, mercury and lead resistance. However, the results only confirmed zinc and cadmium resistances. Mercury resistance could not be determined as the mutants were not sensitive to mercury and therefore, a phenotype could not be established. Lead was not tested at all.

Due to time constraints, there was a small part of the sequence that was not obtained. In future, the entire sequence should be obtained based on the present sequence. From the map in figure 6, it is apparent that digestion of the mutants' genomic DNA with *EcoR* I would be important in achieving this. Once the whole sequence is obtained, it would be important to clone it back into its original host, and then perform more MICs to find out whether it restores the observed phenotype to *Enterobacter* sp. YSU. Eventually, using this sequence as a starter sequence, the entire genome of *Enterobacter* sp. YSU could be sequenced.

CHAPTER V

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