Cloning of Copper resistance gene (copA) that Presence in Novel Genomic Island of *Acinetobacter baumannii* A92

Suhad Saad* ,Alice k. Melconian ,Ali H.Ad'hiah, kumar Rajakumar
Department. of Biotechnology, College of Science, University of Baghdad, Baghdad-Iraq

Abstract

This study was aimed to detect weather *copA* gene(copper resistance gene) presence in *A.baumannii* A92 genome(AbaR genomic islands). The full genomic sequence of *A.baumannii* A92 not published in NCBI genome similarity was detected between two strains so the sequence of *A.baumannii*IS-116(Genebank-AMGF0100000.1) was used to design the primers that were used for amplify of *copA* gene of *A.baumannii* A92.

Two primers contain two sites for restriction enzymes (*KpnI,XohI*) and PWSK29 vector were used in the cloning, double digestion has been performed for vector and gene. Then the re-ligation was completed to form recombinant molecule,after that, transformation have been performed for the recombinant molecule by using chemical competent *E.coli* $DH_5\alpha$. Finally ,the transformant cells were incubated for 16-18hr at 37°C, the white positive colony that contain recombinant vector was appeared .

After that, the success of cloning was confirmed by using colony PCR method for white colony by using copA-F with M13-R(universal primer) primers ,the results of colony PCR confirmed the presence of insert gene by appearing of inserted band.

Key words*: A.bauamnnii* A92, *copA* gene ,copper compound, cloning copper gene.

كلونه جين النحاس المتواجد في الجزيرة الوراثية الفريده المتواجدة في بكتريا *Acinetobacter baumannii* A92

سهاد سعد محمود*، اليس كريكور اغوب، علي، حسين دحيه، كومار راجا كومار
قسم التقنيات الأحيائية ،كليه العلو م، جامعه بغداد، بغداد العراق

الخلاصة

اجريت الدراسه للكشف عن تواجد جين مقاومه عنصر النحاس في موروث (جينوم) بكتريا *Acinetobacter baumannii* A92 .بسبب عدم تواجد التتابعات الكاملة لجينوم *A.baumannii* A92 في موقع NCBI لذلك تم الاعتماد على تتابعات بكتريا *A.baumannii*IS-116 Gene bank– (AMGF0100000.1) حيث وجد تشابه بين تتابعات المادة الوراثية للعزلتين لتصميم البادئات التي استخدمت في تضخيم الجين .حيث تم تصنيع بادئين يحتويان على موقعين للأنزيمات القاطعة (*KpnI,XohI*) وكذلك تم استخدام الناقل PWSK29 كناقل استنسال ،بعد اكمال عمليه الهضم للجين المراد استنساله وناقل الكلونه تم اجراء تفاعل الربط بين الجين والناقل لتكوين الجزيئه الهجينه ثم بعد ذالك تم ادخال الجزيئة الهجينة الى خلايا بكتريا *E.coli* DH5α المؤهلة بواسطه عمليه التحول (transformation) واخيرا تم حضن الخلايا البكتريه المتحولة لمده 16–18 ساعه في 37 م وبعد انتهاء مده الحضن تم الحصول على

*E-mail:suhadsaad@yahoo.com

خلايا بيضاء اللون الحاملة للجزيئة الهجينة .للتأكد من نجاح عمليه الاستنسال وتواجد الجزيئة الهجينة ، تم استخدام تضاعف السلسة للخلايا باستخدام بادئ cop-R والبادئ العام M13-R(universal primer) وقد بينت نتائج التفاعل لل colony-PCR على الجل وجود الجين المحشور من خلال ظهور حزمه (2484 زوج قاعدي) الجين المحشور.

Introduction

Heavy metals are toxic to bacteria. They have been used as antimicrobials in numerous applications for centuries[1]. The heavy metals have been used in water treatment, dietary supplements, clinical treatments, dental amalgams and to create antimicrobial coatings and products[2].

The oldest recorded medical use of copper is mentioned in the Smith Papyrus, one of the oldest books known. This Egyptian medical text, written between 2600 and 2200 B.C., describes the application of copper to sterilize chest wounds and drinking water. Greeks, Romans, Aztecs, and others also used copper or copper compounds for the treatment of such ailments as headaches, burns, intestinal worms, and ear infections and for hygiene in general. In the 19th century, a new awareness of copper's medical potency was spawned by the observation that copper workers appeared to be immune to cholera in the 1832 and subsequent outbreaks in Paris, France. The use of copper in medicine became widespread in the 19th and early 20th centuries, and a variety of inorganic copper preparations were used to treat chronic adenitis, eczema, impetigo, scrofulosis, tubercular infections, lupus, syphilis, anemia, chorea, and facial neuralgia[3].

Bacteria, yeasts, and viruses are rapidly killed on metallic copper surfaces, and the term "contact killing" has been coined for this process. The antimicrobial activity of copper and copper alloys is now well established, and copper has recently been registered at the U.S. Environmental Protection Agency as the first solid antimicrobial material. In several clinical studies, copper has been evaluated for use on touch surfaces, such as door handles, bathroom fixtures, or bed rails, in attempts to curb nosocomial infection .

The spread of antibiotic resistance through selective pressure began and today has made antibiotic-resistant bacteria ubiquitous in hospitals, nursing homes, food processing plants, and animal breeding facilities. This has raised the need for different approaches to keep pathogenic microorganisms at bay. One such alternative is the use of copper surfaces in hygiene-sensitive areas, Recently ,bacteria evolved a range of mechanisms to protect themselves from the toxic effects of copper ions by extracellular sequestration of copper ions, relative impermeability of the outer and inner bacterial membranes to copper ions, metallothionein-like copper-scavenging proteins in the cytoplasm and periplasm, and active extrusion of copper from the cell and by presence of *copA* gene in their genome[4],so the goal of this study was to detect if this strain has ability to resistance copper or not by detecting the presence of copper resistance gene .

Material and methods

Bacterial strain

Acinetobacter baumannii A92that used in this study was obtained as stock in freeze- glycerol solution from University of Leicester/UK. It was refreshed by streaking on LB agar .Then ,it was incubated at 37C° for 18 h and followed by taken of single colony that then was cultured in LB broth at 37C° for 18h by shaking at 200 rpm .Finally, the sample was cultured on LB agar plate and after incubation kept at 4˚C for use in experiments.

Chromosomal DNA extraction

Chromosomal DNA from *A.baumannii* A92 was isolated using the Wizard genomic DNA purification kit (Promega, USA) by using the protocol for bacterial cells according to the manufacturer's instructions.

Cell lysis : 500µl cell suspension (overnight culture) was added to 1.5ml microfuge tube on ice, then , the sample was centrifuge at 13,000-16,000xg for 5 sec to pellet cells then carefully,the supernatant was removed by a pipette . After that ,300µl cell lysis solution was added and mix by pipette it up and down until cells are suspended. The sample was incubated at 80°C for 5min to lyse cells RNase treatment: 1.5 µl RNase A solution was added to the cell lysate and then the sample was mixed by inverting the tube 25 times and then the sample was incubated at 37°C for 15-60 mins.

Protein precipitation: the sample was cooled to room temp by placing it on ice for 1 min then 100µl protein precipitation solution was added to the cell lysate, after that ,the sample was mixed by vortex,

then ,the sample was centrifuged at 13000-16000 xg for 3 mins lead to the precipitate proteins that lead to form tight pellet .

DNA precipitation : the supernatant that contains DNA (leaving behind the precipitation protein pellet) was poured in to a clean 1.5 ml microfuge tube containing 300ml 100% isopropanol ,then the sample was mixed by inverting gently 50 times. After that the sample was centrifuged at 13000-16000 xg for 1 min ,the DNA should be visible as small white pellet. Then ,the supernatant was discard and then the tube was drained on clean absorbent paper ,following that , 300ml of 70% ethanol was added and then the tube was inverted several times to wash the DNA, after that , the sample centrifuged at 13000-16000 xg for 1 min ,then the ethanol pellet was poured and then inverted and the tube was drained on clean absorbent paper and allowed to air dry 5-10 mins.

DNA hydration : 50µl of DNA hydration solution was added to the sample ,and then DNA was rehydrated by incubating sample for 1hr at 65°C and or overnight at room temp ,Finally , DNA was stored at 4° C ,long term storage at -20° C or- 80°C.

Extraction of Plasmid DNA

Plasmid extraction was performed in accordance with the manufacturers' instructions using Miniprep kit (Sigma). Briefly, *E. coli* DH5α, harboring the desired plasmid was streaked onto LB agar plates supplemented where appropriate with antibiotics(ampicillin100µg/ml) and incubated overnight at 37°C. Single colonies were transferred to 5 ml of LB broth containing appropriate antibiotics (ampicillin100µg/ml) and cultured overnight with shaking at 200 rpm. The following day, 1-5ml of an overnight culture of bacteria was pelleted by centrifuging at 12000 xg for 1min and the supernatant was discarded .After that ,the bacterial pellet was re-suspended with 200µl of re-suspension solution

RNase A, then the sample was mixed by vortex or pipette up and down to throuly re-suspend the cells until homogeneous .then, the re-suspended cells were lysed by adding 200µl of the lysis buffer, immediately the contents were mixed by genital inversion (6-8 times) until the mixture becomes clear and viscous. After that, the cleared lysate was transferred to miniprep binding Column(that prepare by adding 500µl of the Colum preparation solution and then centrifuge at 12000 xg for 1 min) ,after that ,the sample was centrifuged at 12000 xg for 1 min and then the flow –through liquid was discard.

Then, 500µL of wash solution was added to the Colum and centrifuged at 12000 xg and then the flow –through liquid was discard , following wash solution II was added and then the flow-through liquid was discarded, centrifuge of column was repeated at 12000 xg for 1 min to remove excess ethanol. Finally the column was transferred to a fresh collection tube was transferred and then 100 µl of nuclease-free deionized water was added and centrifuged at 12000 xg for 1 min ,now the DNA is present in the elute and finally stored at -20°C.

The gene was amplified by taking about 250 bp upstream and downstream of gene(amplify sequence region (77744-80211) ,then two primers with restriction enzyme site of *XhoI* (CTCGAG) *KpnI* (GGTACC) that produced a sticky ends(*CopA*-F-**AGTGGTACC** TTGAACCTAAGGCACCTTTACC, *CopA*-R- **TGACTCGAG** CATAGCCAGCACGACCACAG) was designed and used in amplify process by using of KOD -PCR table-1 procedure as follows:

Table-1-KOD PCR program for gene amplifying

program	REACDTION MIX	1 X µl	2x µl	
1-95ºC for 2 min 2-95ºC for 20sec 3-tm for primers (60ºC)for 10 sec 4-70ºC for 45sec 5-cycle 2 for more 34 times 6-72ºC for 3 min 7-hold in 15 ºC	nH_2O(ultrapure water) $dNTP_S$ (10Mm/µl) $MgSO_4$ Cop-A-F+R 10 X Buffer DNA template KOD polymerase	37 0.5 3.0 1.5(each) 5.0 1.0 0.5	74.0 1.0 6.0 3.0 10.0 2.0 1.0	Template DNA 1:10

After amplifying of gene ,the product of PCR was purified and then restriction digestion reaction has been performed for insert and vector according NEB Restriction Enzymes kit .

The PWSK29 and *copA* gene were digested with *KpnI* and *XhoI* enzyme (the plasmid has multiple cloning site has these restriction sites),double digestion was performed table-2 :

Table -2-The amounts of restriction and digestion reaction

Component	Plasmid µl	Insert µl
H_2O	7.5	7.5
10X NEbuffer	5	5
DNA	35(100ng)	35(102.4ng)
100x BSA	0.5	0.5
KpnI	1	1
XhoI	1	1
Total	50	50

All digestion reactions were performed at 37ºC for overnight .The digested plasmid and insert were purified by Gene flow Q-Spin Gel Extraction/PCR Purification Kit.

Ligation reaction:

Promega ligation kit was used for ligation of vector with the insert. It was recommended to use a molar ratio of 1:10 vector to insert, the following reaction was performed in a sterile microcentrifuge tube table-3.

Table-3-The component quantities of ligation reaction

Reagent	**Amount**
Water	10.8 µl
Vector	5µl(50ng)
Insert	2µl(66ng)
Ligase 10 x Buffer	2µl
T4 DNA Ligase:	0.2 u/µl
Total	20µl

The sample was Incubated at room temperature on bench for 1 hour, then in 4°C overnight. The next day, the ligation reaction was taken from the fridge and leave on bench for 1 hour to come to room temperature. Then chemical transformation has been performed by using of chemical component *E. coli* DH5α cells .

Results and discussion

To detect the presence of copper resistance gene in *A.baumannii* A92,two primers were designed with sites of two restriction enzymes that used to amplify the gene(used *A.baumannii*IS-116-Genebank-AMGF0100000.1) by KOD procedure . PWSK29 vector was isolated from *E.coli*DH5α that resistance to ampicillin,the result of KOD PCR and gel electrophoresis were shown in figure-1 and 2 :

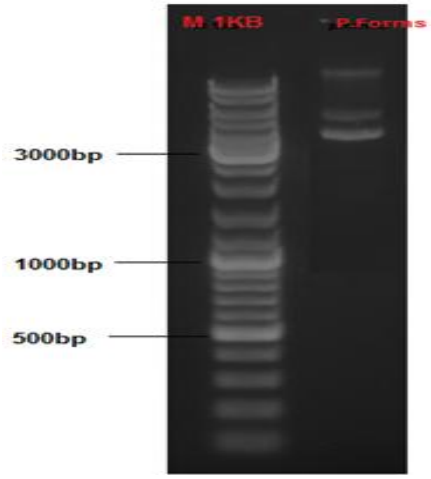


Figure 1-PWSK29 vectors after isolation and purification from *E.coli* DH5α on 1% agarose, at 100 V for 1 hr.

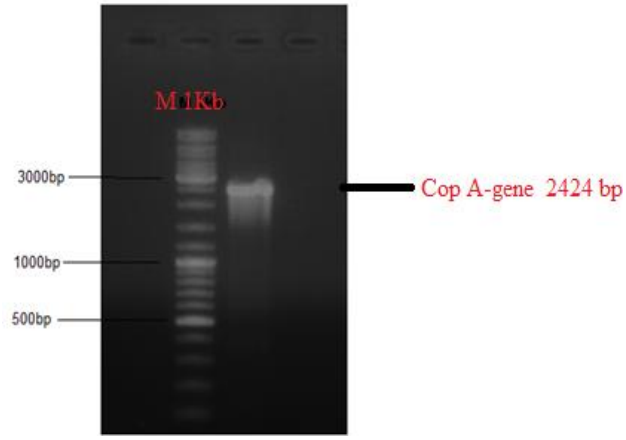


Figure 2-Agarose gel electrophoresis of amplified *Cop A-gene* of *A.baumannii* A29 on 1% agarose at 11 V for 1 hr (2424bp).

The results in figure 2 was confirmed the present of cooper resistance gene in *A.baumannii* A92 that resulted high resistance ability of bacteria against copper .The results in figure-3 , three forms of PWSK29 vector were appeared include linear ,open coiled and supercoiled that differ in their movement in gel that results from their differentiation in their shapes[5].

After isolation and purification of vector and *copA* gene ,restriction digestion reaction have been performed overnight by incubation at 37C° ,then ,the purification of the vector and gene have been performed and the results of gel electrophoresis showed below figure-3

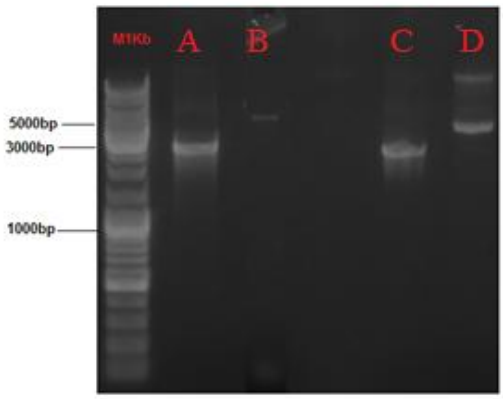
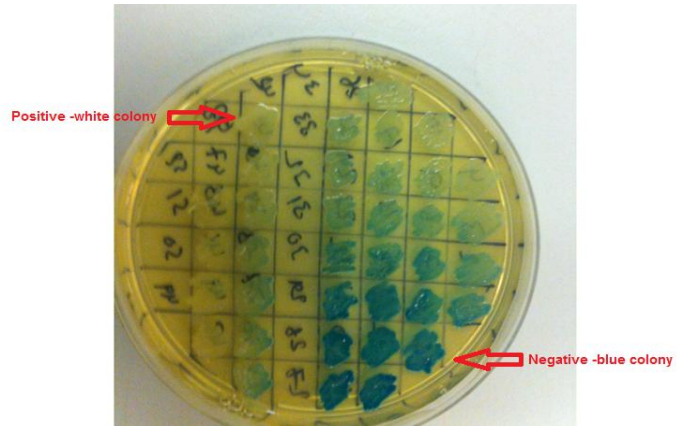


Figure 3-Agarose gel (1%) electrophoresis of A and B digested vector and gene C and D un digested vector and gene at 100 V for 1 hr.

The digest PWSK29 vector and insert(*copA*) were relighted .The recombinant molecule that contain desired gene transform to chemical competent *E.coli*DH5α.The transform *E.coli*DH5α cells were cultured overnight in LA plates that contain X-gal and ampicillin antibiotics that help the detection of bacterial cells that contain recombinant molecule .White colonies were resulted from insertion of desired gene in *Lac Z* gene(β-galactosidase) that work on X-gal(5-bromo-4-chloro-3-indolyl-β-D-ggalactopyranoside),β-galactosidase enzyme which cleaves the β-glycosidic bond in D-lactose. X-gal, when cleaved by β-galactosidase, yields galactose and 5-bromo-4-chloro-3-hydroxyindole. The latter then spontaneously dimerizes and is oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, an intensely blue product which is insoluble , while when *lac z* gene disrupted by an insert result non-functional *LacZ* gene, and are unable to metabolize Xgal, therefore produce white colonies. Thus, the white colonies are the ones of interest[6] figure-4 .



Figuer-4 –Lurea agar contains Xgal,Ampicillin antibiotic showed
Transformat*E.coli*DH5α Blue colony-negative
Transformat*E.coli*DH5α white colony-positive

After transformation of *E.coli*DH5α and got positive white colony that contain recombinant vector ,Colony PCR method have been performed to screened for desired insert in white cells ,this method have been performed by using copA-R primer and M13-F(universal primer) ,the results was appeared expected band size 2484bp figure-5 :

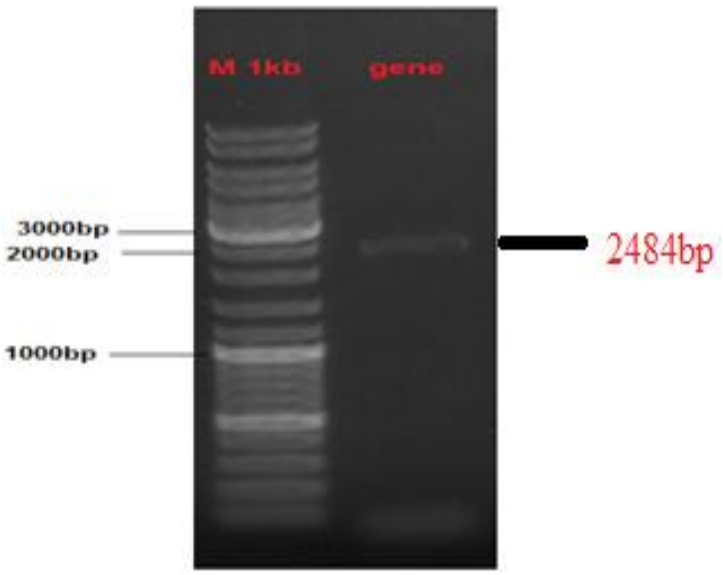


Figure 5-Gel electrophoresis of Colony PSR product of white colony *E.coli* DH5α that gave expected band size 248bp on 1% agarose ,at 100 V for 1 hr.

Reference:

1. Gittard, S.D., Perfect, J.R., Monteiro-Riviere, N.A., Wei, W., Jin, C. and Narayan, R.J., **2009**. Assessing the antimicrobialactivity of zinc oxide thin films using disk diffusionand biofilm reactor. *Appl Surf Sci*, 255:5806-5811.
2. Rajendran, R., Balakumar, C., Ahammed, H.A.M., Jayakumar, S., Vaideki, K. and Rajesh, E.M., **2010** Use of zinc oxide nano particles for production of antimicrobial textiles. *Int J Eng Sci Technol*., 2:202-208.
3. Adams, M.D., Goglin, K., Molyneaux, N., Hujer, K.M., Lavender, H., Jamison, J.J., MacDonald, I.J., Martin, K.M. , Russo, T., Campagnari , A.A., Hujer, A.M., Bonomo, R.A. and Gill, S.R. **2008**.Comparative genome sequence analysis of multidrug-resistant *Acinetobacter baumannii*. *J .Bacteriol.*, 190:8053-8064
4. Grass, G., Rensing, C. and Solioz, M. **2011**.Metallic Copper as an Antimicrobial Surface. *Appl .Environ. Microbiol*., 77: 1541-1547.
5. Schmidt,T.,Friehs,K.,Schleef,M.,Voss,C.,Flaschel,E.**1999** Quantitative analysis of plasmid forms by agarose and capillary gel electrophoresis.Analytical *Biochemistry*,274:235-240
6. Sweeney, S.T., Hidalgo, A., De belle, J.S. and Keshishian, H., **2012**. *X-Gal Staining of the Central Nervous System in Adult Drosophila*. Cold Spring Harbor Protocols, 2 :067405.