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Detection of plasmids and curing analysis in copper resistant bacteria *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4

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Abstract. *Irawati W, Yuwono T, Rusli A. 2016. Detection of plasmids and curing analysis in copper resistant bacteria Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4. *Biodiversitas 17: 296-300. Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4 were copper resistant bacteria. The aims of the study were to establish correlation between bacterial resistance and the presence of plasmid and to prove the presence of gene that encodes resistance to copper in plasmid. Plasmid curing was carried out by the addition of ethidium bromide, acridine orange, and SDS in Salt Base Solution broth medium. Detection of copper resistance gene in plasmid was carried out by PCR method using *CopA* primer. The study showed that plasmid isolation has been successfully performed in *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4. The size of plasmid was approximately more than 21 kb. The most effective curing treatment in *Acinetobacter* sp. IrC1 was 600-700 µg/ml ethidium bromide that reduced up to three times of copper resistance after curing treatment. Meanwhile, copper resistance in *Acinetobacter* sp. IrC2 and *Cupriadus* sp. IrC4 decreased four times after curing treatment using 150-200 µg/mL acridine orange and 3000-3500 µg/mL SDS, respectively. The decrease of copper resistance following plasmid curing treatment suggested that copper resistance gene was encoded by the plasmid. The amplification of *CopA* gene in the plasmid showed the presence of single band DNA with approximately 1.8 kb. The finding on copper resistance present in the plasmid may open a wider application of bacteria as copper bioremediation agent.

Keywords: Copper, curing, gene, plasmid, resistance
Abbreviations: Sodium dodecyl sulphate (SDS), Ethidium bromide (EtBr), Acridine orange (AO)

INTRODUCTION

The presence of toxic heavy metal contaminants in aqueous streams, arising from the discharge of untreated metal containing effluents into water bodies, is one of the most important environmental issues (Rehman et al. 2008). Exposures of toxic heavy metals make the cells of microorganisms develop resistance mechanisms and metal-ion homeostasis (Aspassia et al. 2007). Microbial populations in metal polluted environments adapt to toxic concentrations of heavy metals and become metal resistant (Prasenjit and Sumanthi 2005). Metal-tolerant bacteria could survive in these habitats and could be isolated and selected for their potential application in bioremediation of contaminated sites (Piotrowska-Seget et al. 2005).

Heavy metals are groups of pollutants, which are not biodegradable and tend to accumulate in living organisms (Kobya et al. 2005). Since heavy metal ions can not be degraded or modified, there are few mechanisms of bacteria for heavy-metal resistance systems such as binding to the cell surface, influx and efflux, accumulation, detoxification of toxic metals to less toxic form, the use of metallothionein protein, and combination of two or three mechanisms mentioned (Aspassia et al. 2007). The well-known copper resistant strain of *Pseudomonas syringae* pv. *tomato* is able to accumulate copper as resistance mechanism to copper. Molecular analysis of the copper resistance nature on *P. syringae* pv. *tomato* revealed an operon Copper (*Cop*) located in the plasmid, namely structural gene *CopA*, *CopB*, *CopC*, and *CopD* as well as regulator gene *CopR* and *CopS*. Each protein functioned specially but *CopA* protein was apparently the most responsible protein in the resistance of *P. syringae* pv. *tomato* (Cha and Cooksey 1991).

The genes conferring copper resistance in bacteria are usually present in plasmids or chromosome. Plasmid is one of the several environmental and genetic factors that carry the resistance property against a specific drug or number of drugs in bacteria. Curing of a cryptic plasmid from a bacterial strain is a method to substantiate the relationship between a genetic trait and carriage of that specific trait in the plasmid. Various methods involving chemical and physical agents have been developed to eliminate plasmid (Crosa et al. 1994).

Acinetobacter sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4 were copper resistant bacteria isolated from activated sludge in an industrial wastewater treatment plant in Rungkut-Surabaya, Indonesia. Bacterial isolates showed resistance mechanism by copper accumulation inside cells (Irawati et al. 2012). The present study was a preliminary effort to observe the curing efficiencies of acridine orange, ethidium bromide, and SDS on copper resistant bacterial isolates to establish a correlation between plasmid elimination and subsequent loss of copper resistance, and also to prove the presence of the gene that encodes resistance to copper.

MATERIALS AND METHODS

Growth media

Bacteria were grown in Salt Base Solution (SBS) broth containing the following (per liter): K_2HPO_4 1.5 g; KH_2PO_4 0.5 g; $(NH4)_2SO_4$ 0.5 g; $Mg_2SO_4.7H_2O$ 0.2 g, supplemented with appropriate concentration of copper sulfate and in medium without copper. Cells were incubated at 37°C on a shaker 200 rpm. Growth was monitored by measuring optical density at 600 nm (Irawati et al. 2013).

Plasmid DNA isolation

Bacterial isolates were tested for the presence of plasmids using alkaline lysate method with slight modification (Sambrook et al 1989). Bacterial colony was inoculated into 5 mL SBS medium containing appropriate concentration of copper sulfate and incubated overnight on a shaker 150 rpm at 37°C. Overnight culture (1.5 mL) was transferred into a microfuge tube and centrifuged at 8,000g for 2 minutes. Bacterial pellets were resuspended in 100 µl of solution which contained 25 mM Tris-HCL, 10 mM EDTA, 50 mM glucose and 10 µl of 2 mg/mL lysozyme, then incubated on ice for 30 minutes. After incubation, 200 µl of solution II (2 N NaOH and 10% SDS) was added. The tube was mixed by inverting it for several times and then incubating it on ice for 10 min. A 150 µl 7.5 M potassium acetate solution was added to the tube and gently mixed by inverting the tube 6 times. It was then incubated on ice for 60 min, and centrifuged at 8,000g. The supernatant was transferred into a new microfuge tube and added with phenol (v/v), then gently mixed by inverting the tube several times and centrifuged at 8,000g. Top aqueous layer was transferred into a new microfuge tube and added with 1 mL ethanol absolute, mixed well, and stored at-20 ^{0}C for overnight. The aqueous was centrifuged at 8,000g, then ethanol absolute was discarded and washed with 1 mL of 70% ethanol. The pellet was dried and resuspended with 30 µl TE. Plasmid DNA was analyzed by electrophoresis on 1% (w/v) agarose gel in TAE buffer 0.5 µg/mL EtBr.

Plasmid curing

Plasmid was cured essentially as described by Miller (1972). Chemical agents used in this research were EtBr (500-800 µg/mL), AO (25-125 µg/mL), and SDS (500-5,000 µg/mL). Cells (approximately 0.1 mL of a 10^{-5} dilution of a fresh overnight) were inoculated into 5 mL SBS broth, pH 7.6, containing appropriate concentration of curing agents. In similar manner, a control culture without curing agents was prepared. The overnight cultures were plated by dilution on copper indicator plates and the single colonies were observed. The presence and absence of single colonies in medium containing 4 mM $CuSO_4$ were observed. Cured cells were tested to verify the loss of the plasmid and its resistance to copper by plasmid DNA isolation. The presence of plasmid was analyzed by electrophoresis on 1% (w/v) agarose gel in TAE buffer 0.5 µg/mL EtBr.

Amplification of copper resistance gene

Copper resistance gene was amplified as described by Rusli (2012). Amplification was carried out by using *CopA* primers encoding copper resistance in *Acinetobacter* sp. Amplification was accomplished by thermocycler with 1x PCR buffer with 4.5 mM $MgCl_2$, 200 µM each dNTP's (Promega), Taq polymerase 1U/µL (Promega), 0.1 µM forward primer of *CopA* (5'-TAG AGC AGA TGG CAA TGA ATC GCC CAT-3') and reverse primer of *CopA* (5'-AGT TGG AAG AGG GGG ATG AAG CTG TTA TTC-3'), 1 µL template DNA, and ddH_2O to a final volume of 60 µL. PCRs were performed with 10' initial denaturation at 95°C, 35 cycles of 1' denaturation at 95°C; 1' annealing at 64°C, 1,5'; and 1' extension at 72°C, 2' final extension at 72°C, and storage at 4°C. *CopA* gene was analyzed by 1% agarose gel electrophoresis by comparison with λ DNA/*Hind*III and 1kb markers. The *CopA* gene was analyzed by electrophoresis on a 1% (w/v) agarose gel in TAE buffer with 0.5 µg/mL EtBr.

RESULTS AND DISCUSSION

Plasmid DNA isolation

Isolation of plasmid DNA from bacterial isolates revealed the presences of single plasmid. The size of the plasmid was approximately more than 21 kb (Figure 1).

Plasmid isolation has been successfully performed in *Acinetobacter* sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4. Plasmid are extra-chromosomal genetic elements that can range in size from several hundred base pairs to several thousand kilobases (Zholgharnein et al. 2007). Based on the illustrated sizes, the bacterial isolates had large plasmid size. Different plasmids vary considerably in their property to be cured, and not necessarily depending upon properties of specific plasmid (Zaman et al. 2010). Plasmid can be eliminated by curing agents which can be used to display the role of R-plasmid in drug resistance. The techniques used to promote curing include exposing the host strain to elevated temperatures, use of chemical agents such of intercalating dyes (acridine orange, ethidium bromide, SDS, thymidine starvation and exposure to UV radiation (Clowers 1972).

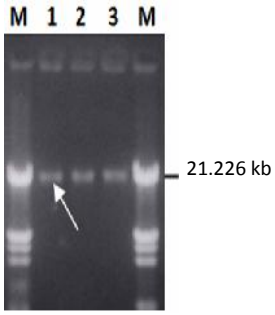

Figure 1. Plasmid DNA profile of (1) *Acinetobacter* sp. IrC1, (2) *Acinetobacter* sp. IrC2, and (3) *Cupriavidus* sp. IrC4. (M) Marker DNA lambda *Eco*R1/*Hin*dIII.

Plasmid curing

Plasmid curing in this study was carried out by AO, EtBr, and SDS. After curing treatment, each cured colonies of bacterial isolates was isolated and retested for copper resistance by growing in medium containing 4 mM $CuSO_4$. The effect of plasmid curing on the copper resistance of bacterial isolates was shown in Table 1.

Table 1 showed that plasmid curing by appropriate concentrations of curing agent resulted in colony inhibition so that there was no growth of colony on medium containing copper. The curing treatment was successfully done when cured cells which still allowed growth in medium containing the highest concentration of curing agent could not grow in medium supplemented with copper varied among plasmids of bacterial isolates. The effectiveness of each of bacterial isolates to curing agents plasmid of *Acinetobacter* sp. IrC1 could not be cured efficiently by 500 µg/mL SDS whereas *Acinetobacter* sp. IrC2 could not be cured efficiently by 250 µg/mL AO and SDS up to 2,000 µg/mL. Meanwhile, *Cupriavidus* sp. IrC4 could not be cured efficiently by treatment with 250 µg/mL EtBr and AO up to 225 µg/mL.

According to Horn and Korn (1979) as no universally effective curing agent has yet been identified, curing experiments are generally conducted on trial and error basis, both with respect to the choice of the curing agent and the culturing conditions used. Some curing agents works in a non-specific way by damaging and stressing out the cells, while some seem to act much selectively. Table 1 showed that the effectiveness of curing treatment in *Acinetobacter* sp. IrC1 was 600-700 µg/mL EtBr and 200 µg/mL AO. Meanwhile, the effectiveness of curing treatment in *Acinetobacter* sp. IrC2 and *Cupriavidus* sp. IrC4 were 150-200 µg/mL AO and 3000-3500 µg/mL SDS, respectively. The correlation between plasmid elimination and subsequent loss of copper resistance was shown in Table 2.

Table 2 showed that the plasmid elimination was accompanied by drastic changes in copper resistance in bacterial isolates. Copper resistance in *Acinetobacter* sp. IrC1 was reduced from 8 mM to 3 mM when cured by 700 µg/mL ethidium bromide and was reduced from 8 mM to 4 mM $CuSO_4$ when cured by 200 µg/mL acridine orange. Copper resistance of *Acinetobacter* sp. IrC2 was reduced from 9 mM to 2 mM after treatment with 200 µg/mL acridine orange, meanwhile copper resistance in *Cupriavidus* sp. IrC4 was reduced from 11 mM to 3 mM when it was cured by 3,500 µg/mL SDS.

The cured colonies were analyzed for the presence of plasmid DNA on agarose gels (Figure 2). It can be seen that there was difference of plasmid profile between cured strain and wild strain. The cured plasmid of *Acinetobacter* sp. IrC1 could not be detected clearly after treated by 200 µg/mL of acridine orange (lane 2 and 3). Meanwhile the cured plasmid of *Cupriavidus* sp. IrC4 was having the different migration with wild plasmid after cured by 3,500 µg/mL SDS (lane 11 and 12). It might be caused by intercalation between two bases of plasmid DNA by curing agent so that the plasmid could not be replicated well. This curing treatment accompanied by drastic changes in resistance of bacteria indicated that gene encoding copper resistance in *Acinetobacter* sp. IrC1 and *Cupriavidus* sp. IrC4 were located in plasmid.

Table 1. The effect of curing treatment on the growth of bacteria in medium containing 4 mM $CuSO_4$

Curing agent	(µg/mL)	Amount of colonies					
		Acinetobacter sp. IrC1		*Acinetobacter* sp. IrC2		*Cupriavidus* sp. IrC4	
		Without copper	With copper	Without copper	With copper	Without copper	With copper
Etidium bromida	250	> 100	> 100	> 100	> 100	> 100	> 100
	500	32	15	-	-	-	-
	600	21	-	-	-	-	-
	700	2	-	-	-	-	-
	800	-	-	-	-	-	-
Acridine orange	25	> 100	> 100	> 100	> 100	> 100	> 100
	50	> 100	> 100	> 100	33	> 100	> 100
	75	> 100	18	38	16	> 100	> 100
	100	> 100	15	27	8	> 100	> 100
	125	22	8	28	5	> 100	> 100
	150	23	5	8	-	> 100	> 100
	175	21	4	5	-	> 100	> 100
	200	2	-	4	-	> 100	> 100
	225	-	-	-	-	> 100	> 100
SDS	500	> 100	> 100	> 100	> 100	> 100	> 100
	1,000	-	-	> 100	> 100	> 100	> 100
	1,500	-	-	> 100	> 100	> 100	> 100
	2,000	-	-	> 100	> 100	> 100	> 100
	2,500	-	-	-	-	> 100	> 100
	3,000	-	-	-	-	52	-
	3,500	-	-	-	-	2	-
	4,000	-	-	-	-	-	-

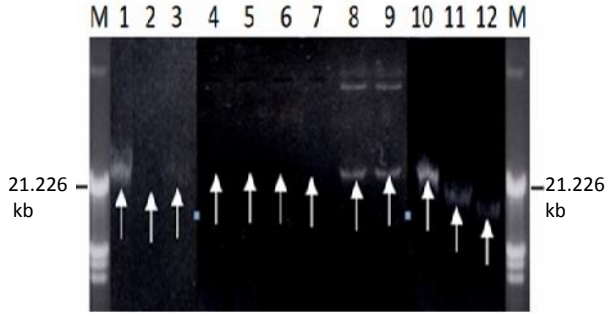

Figure 2. Plasmid DNA profile of bacterial isolates without curing and after curing treatment, (Lane 1: *Acinetobacter* sp. IrC1 plasmid DNA, 2 and 3: acridine orange cured, lane 8 and 9: *Acinetobacter* sp. IrC2 plasmid DNA, 4-7 acridine orange cured, lane 10: *Cupriavidus* sp. IrC4 plasmid DNA, 11-12 SDS cured). (M) Marker DNA lambda *Eco*R1/*Hind*III.

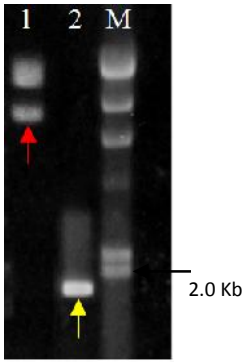

Figure 3. Plasmid and PCR product of *Acinetobacter* sp. IrC1. Note: 1: *Acinetobacter* sp. IrC1 plasmid, 2: *CopA* gene of *Acinetobacter* sp. IrC1 and M: lambda DNA/*Hind*III marker (Promega). Arrows indicate the presence of plasmid and *CopA* gene.

Table 2. The effect of plasmid curing on the copper resistance to isolates bacteria.

Treatment	Concentration										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Acinetobacter sp. IrC1											
Without curing	+	+	+	+	+	+	+	+			
EtBr (700 µg/mL)	+	+	+	+							
A O (200 µg/mL)	+	+	+								
Acinetobacter sp. IrC2											
Without curing	+	+	+	+	+	+	+	+	+		
A O (200 µg/mL)	+	+									
Cupriavidus sp. IrC4											
Without curing	+	+	+	+	+	+	+	+	+	+	+
SDS 3500 µg/mL	+	+	+								

Note: (+) resistant to copper

Plasmid DNA profile of bacterial isolates without curing and after curing treatment can be seen at Figure 2. Figure 2 showed that the cured plasmid of *Acinetobacter* sp. IrC2 could not be detected by gel electrophoresis after treated by 200 µg/mL acridine orange (lane 4 and 7). Concomitant to this loss of plasmid, the cured cell became sensitive to copper. This clearly showed relationship between the loss plasmid DNA and the loss of copper resistance properties. Cured strain became sensitive to copper after curing treatment indicated the possible plasmid borne nature of gene encoding resistance to copper. The loss of copper resistance in cured strain may be because of mutation as a result of incubation in the presence of curing agent (Raja and Selvam 2009).

CopA gene amplification

The amplification of *CopA* gene in the plasmid showed the presence of single band DNA with approximately 1.8 kb similar to the length of *CopA* gene in *Pseudomonas syringae* pv. *tomato* (Figure 3).

CopA gene which has been successfully amplificated in plasmid of *Acinetobacter* sp. IrC1 indicated that copper resistance determinant of the bacterial isolate encoded in plasmid. It was previously reported that copper resistance mechanism of *Acinetobacter* sp. IrC1 was facilitated through the bioaccumulation of copper inside the cell which was marked by the alteration in the color of the colonies into blue in high concentration of copper similar to *Pseudomonas syringae* (Irawati et al. 2012). The *CopA* gene encodes a methionine-rich periplasmic protein capable of binding up to 11 copper atoms in *Pseudomonas syringae* pv. *tomato* (Cha and Cooksey 1991).

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