Korean Journal of Microbiology (2016) Vol. 52, No. 3, pp. 254-259
DOI http://dx.doi.org/10.7845/kjm.2016.6043

pISSN 0440-2413
eISSN 2383-9902

Article

Effect of plasmid curing on the 2, 3-dihydroxybenzoic acid production and antibiotic resistance of *Acinetobacter* sp. B-W

Kyoung-Ja Kim[1]*, Jin-Woo Kim[1], and Yong-Joon Yang[2]

[1]*Department of Life Science and Biotechnology, College of Natural Science, Soonchunhyang University, Asan 31538, Republic of Korea*
[2]*Department of Plant and Food Science, Sangmyung University, Cheonan 31068, Republic of Korea*

Acinetobacter sp. B-W의 2, 3-dihydroxybenzoic acid 생산과 항생제 저항성에 미치는 플라스미드 제거 효과

김경자[1]* · 김진우[1] · 양용준[2]

[1]순천향대학교 생명시스템학과, [2]상명대학교 식물식품공학과

(Received July 27, 2016; Revised August 8, 2016; Accepted August 8, 2016)

ABSTRACT: *Acinetobacter* sp. B-W producing siderophore, 2, 3-dihydroxybenzoic acid (DHB) was analyzed for plasmid content. Strain B-W harbored plasmid of 20 kb in size. Growth at 43°C was effective in producing mutant cured of plasmid of strain B-W. This mutant lost the ability to produce 2, 3-DHB. Formation of siderophore halos on the chrome azurol S (CAS) agar medium was not detected by cured strain B-W. pHs of supernatants of wild type strain B-W and cured mutant grown in glucose and $MnSO_4$ containing medium at 28°C for 3 days were 4.5 and 8.5, respectively. Antibiotic resistance against ampicillin, actinomycin D, bacitracin, lincomycin, and vancomycin was lost in cured mutant. Plasmid curing of strain B-W resulted in drastic reduction of minimal inhibitory concentration (MIC) of several antibiotics. *E. coli* DH5α was transformed with plasmid isolated from strain B-W. The transformant *E. coli* DH5α harbored a plasmid of the same molecular size as that of the donor plasmid. Transformant *E. coli* DH5α produced 2, 3-DHB and contained antibiotic resistant ability. Thus a single plasmid of 20 kb seemed to be involved in 2, 3-DHB production. Genes encoding resistance to antibiotics were also supposed to be located on this plasmid.

Key words: *Acinetobacter* sp. B-W, antibiotic resistance, 2, 3-dihydroxybenzoic acid, plasmid curing

Iron is an essential micronutrient for almost all living organisms. In nature, iron is mainly present in a ferric insoluble state, with reduced biological availability. One of the strategies developed by bacteria to acquire iron under restrictive conditions is the synthesis of low-molecular-mass iron chelators, known as siderophores (Wandersman and Delepelaire, 2004). In our previous report (Kim *et al*., 2015), siderophore produced from *Acinetobacter* sp. B-W was identified as a 2, 3-dihydroxybenzoic acid (DHB). Because of the role that siderophores play in the acquisition of iron (Miethke and Marahiel, 2007; Wencewicz, 2009), the inhibition of siderophore biosynthesis has been identified as a potential target for the development of novel antibacterial agents. Previous reports have shown that 2, 3-DHB is chromosome-borne in *Vibrio anguillarum* (Chen *et al*., 1994). But, anguibactin, siderophore produced from *Vibrio anguillarum* is plasmid encoded (Jalal *et al*., 1989). Curing is the artificial way used by scientists to make microorganisms lose genetic material, mainly plasmids (González *et al*., 1981). Since the loss of plasmids by curing is definitely associated with the loss of certain genes (Driss *et al*., 2011) and consequently the corresponding proteins, this technique was a good tool for determining localization of genes. Most of the genetic determinants that confer resistance to antibiotics are located on plasmids. Plasmid curing occurs naturally through cell division

***For correspondence.** E-mail: kyoungjakim@hotmail.com;
Tel.: +82-41-530-1352; Fax: +82-41-530-1350

or by treating the cells with any chemical or physical agents (Elias *et al*., 2013).The aim of present work is to characterize plasmid curing effect on the 2, 3-DHB production and antibiotic resistance of strain B-W.

Materials and Methods

Microorganisms, media, and culture conditions

In our previous report (Kim *et al*., 2015), the structure of the siderophore produced from *Acinetobacter* sp. B-W grown in modified PGI medium at 28°C was identified as a 2, 3-DHB. Strain B-W was maintained on a semi synthetic minimal medium: Glucose 5 g/L, $(NH_4)_2SO_4$ 0.5 g/L, yeast extract 0.05 g/L, KH_2PO_4 0.2 g/L, $MgCl_2$ 0.6 H_2O 0.2 g/L, $FeSO_4$ 0.005 g/L, agar 20 g/L, pH 7.0. The siderophore production medium for strain B-W and cured strain was a chemically defined one (modified PGI medium, pH 7.0) with the following compositions (in g/L): glucose (5), sodium glutamate (2.5), $MgSO_4 \cdot 7H_2O$ (0.5), K_2HPO_4 (1.0), $MnSO_4$ (0.002).

Curing of plasmid from *Acinetobacter* sp. B-W

Plasmid curing was done by the heat treatment procedure of Zurkowski and Lorkiewcz (1978). For heat curing, overnight nutrient broth cultures were inoculated to approximately 10^6 cells/ml into NB medium. Cultures were incubated at 40, 42, and 43°C and transferred at weekly intervals. Growth at 40, 42, and 43°C was repeated for several times in fresh medium along with occasional monitoring of the plasmid profile in agarose gel. After heat treatment, cultures were spread on NB agar plates, and isolates were purified and checked the loss of 2,3-DHB production in the glucose and $MnSO_4$ containing medium at 28°C. Electrophoresis of plasmid DNA was carried out on 0.7−1.0% (wt/vol) agarose gel, and plasmids were detected after ethidium bromide (EtBr) staining.

Siderophore detection

The Chrome Azurol S (CAS) plates (Schwyn and Neiland, 1987) were used to check the culture supernatant for the presence of siderophore. The presence of siderophore is indicated by a color change from blue to orange. This occurs because iron is removed from the original blue CAS-Fe (III) complex during siderophore production. The amount of 2, 3-DHB, catechol type siderophore of *Acinetobacter* sp. B-W was examined by the Arnow reaction (Arnow, 1937).

Plasmid DNA isolation

Plasmid DNA was isolated from strain B-W grown in ampicillin (100 μg/ml) containing LB medium at 37°C by using plasmid extraction kit (Bioneer). DNA gel electrophoresis was carried out as describe d by Meyers *et al.* (1976) in 0.7% agarose gels.

Transformation of *E. coli* DH5α

Transformation of *E. coli* DH5α with plasmid DNA prepared from strain B-W was achieved following the procedure described by Maniatis *et al*. (1989). A control experiment was carried out with pBR322. Selection of *E. coli* cells transformed with plasmid DNA of strain B-W or pBR322 was made on NA containing ampicillin (100 μg/ml).

Antibiotic susceptibility testing and determination of minimal inhibitory concentrations (MICs)

Antibiotic susceptibility was tested by disc diffusion (Bauer *et al*., 1966) on Mueller-Hinton agar (Bio-Rad). The antibiotics were ampicillin (10 μg), actinomycin D (30 μg), bacitracin (30 μg), lincomycin (30 μg), and vancomycin (30 μg). Plates were incubated at 37°C for 24 h, and diameter of zone of inhibition around each antibiotics-containing disc was measured. The MICs of antibiotics were determined by the method of Steers (Steers *et al*., 1959) with 10^4 CFU per spot on agar after 24 h of incubation. The antibiotic concentrations ranged from 1.0 μg/ml to 1,000 μg/ml. All measurements were performed in duplicates which had identical results in all measurements. The MIC was defined as the minimal concentration of antibiotics that limited growth to an OD_{600} of 0.2 or less after 24 h. MICs were determined in duplicate by broth dilution methodology in microtiter plates according to the general recommendation of the CLSI (2010).

Results and Discussion

Isolation and curing of plasmid from *Acinetobacter* sp. B-W

Isolated plasmid from *Acinetobacter* sp. B-W was analyzed

by agarose gel electrophoresis.

The size of plasmid was 20 kb. The strain B-W was subjected to plasmid curing (Zurkowski and Lorkiewcz, 1978) at 43°C to check the loss of 2, 3-dihydroxybenzoic acid (DHB) production in the glucose and $MnSO_4$ containing medium at 28°C and antibiotic resistance. Plasmid was not detected in the cured strain B-W. Curing at 40 or 42°C of strain B-W was not successful. Transformant *E. coli* DH5α harbored a plasmid of the same molecular size as that of the donor plasmid (Fig. 1). Elevated incubation temperature (up to 42°C), ethidium bromide (0.5 mg/ml), 10% SDS, and acridine orange (0.5 mg/ml) were reported to curing methods to eliminate plasmids from *Vibrio* species (Zhang *et al*., 2012). All these chemical curing agents are known to be harmful and cause health problems to human beings. Precaution steps should be followed strictly prior in handling with these curing agents during experiments. But, chemical curing methods were not successful in strain B-W. When compared with chemical curing agents, physical agent such as elevated growth temperature is less favored in *Vibrio* plasmid curing studies due to its low successful rate. But, elevated growth temperature (43°C) was effective method for curing in strain B-W.

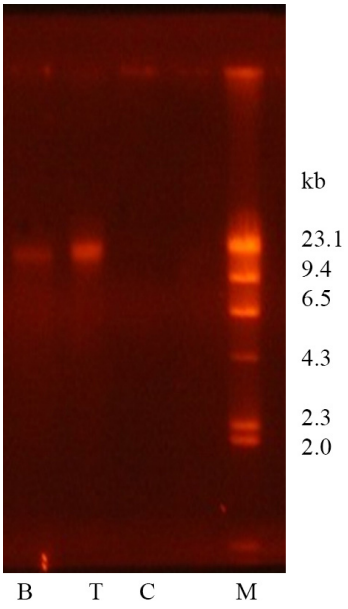

Fig. 1. Agarose gel electrophoresis of plasmid DNA from *Acinetobacter* sp. B-W (B), transformant *E. coli* DH5α (T) and cured strain B-W (C). M, DNA standard marker.

Effect of plasmid loss on growth and 2, 3-dihydroxybenzoic acid production of *Acinetobacter* sp. B-W

Plasmid curing affected the growth rate of strain B-W. Growth rate of cured strain was approximately 57% of strain B-W (Fig. 2). Significant difference was observed in the 2, 3-DHB production of plasmid cured strain B-W, when compared to the parental one. Production of 2, 3-DHB of strain B-W was totally lost by curing (Figs. 3 and 4). Production of siderophore was detected on a CAS agar plate. Orange halos of supernatant from strain B-W and transformant *E. coli* DH5α were 18–24 mm, but that of cured strain B-W was 0 mm (Fig. 3). Catechol type sideropore, 2, 3-DHB was assayed by Arnow test (Arnow, 1937) at 510 nm (Fig. 4). Disappearance of 2, 3-DHB production with concurrent loss of plasmid from strain B-W suggested that 2, 3-DHB biosynthetic genes were plasmid-borne. It was con-

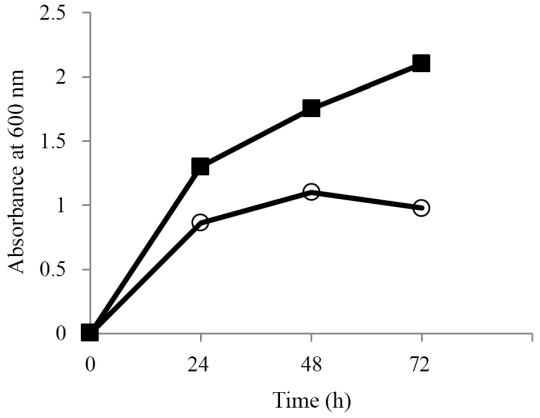

Fig. 2. Growth curve of *Acinetobacter* sp. B-W (■) and cured strain B-W (○) grown in modified PGI medium at 28°C.

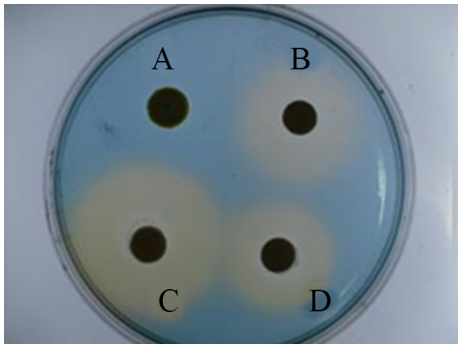

Fig. 3. CAS agar assay. The CAS agar assay was performed on a CAS plate with paper discs those were coated with followings; A, Supernatant of cured strain B-W at 24 h growth; B, Supernatant of strain B-W at 12 h growth; C, Supernatant of strain B-W at 24 h growth; D, Supernatant of transformant *E. coli* DH5α at 12 h growth.

firmed from the result that 2, 3-DHB production was restored in the transformant *E. coli* DH5α. But, 2, 3-DHB production in *Vibrio anguillarum* is reported to be chromosomal mediated (Chen *et al*., 1994).

pH of supernatant of *Acinetobacter* sp. B-W, cured strain B-W and transformant *E. coli* DH5α

As shown in Fig. 5, pHs of supernatant of *Acinetobacter* sp. B-W grown in modified PGI medium at 28°C for 3 days was 4.0. But pH of supernatant of cured strain B-W was 8.5. Loss of 2, 3-dihydroxybenzoic acid (DHB) production of cured strain B-W is likely to have influence on the pH of supernatant. Biosynthetic intermediates of 2, 3-DHB in *Aerobacter aerogenes* and *E. coli* were known as chorismic acid, isochorismic acid, and 2, 3-dihydro-2, 3-dihydroxybenzoic acid (Young and Gibson, 1969). Acidic biosynthetic intermediates and final product of 2, 3-DHB might have affected acidic pH of supernatant of strain B-W.

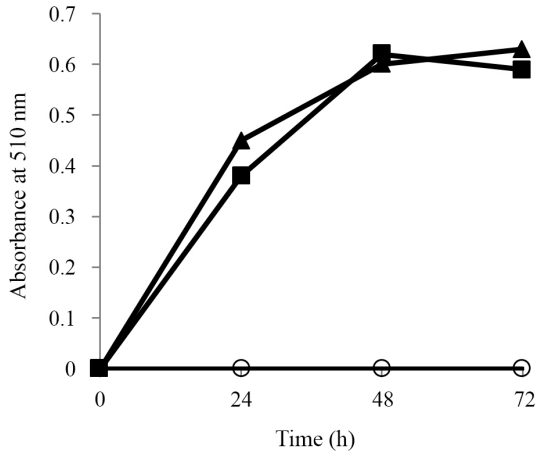

Fig. 4. Production of 2, 3-dihydroxybenzoic acid (DHB) by *Acinetobacter* sp. B-W (■), cured strain B-W (○), and transformant *E. coli* DH5α (▲) during growth. Cells were grown in modified PGI medium at 28 for the determination of 2, 3-DHB concentration by Arnow test.

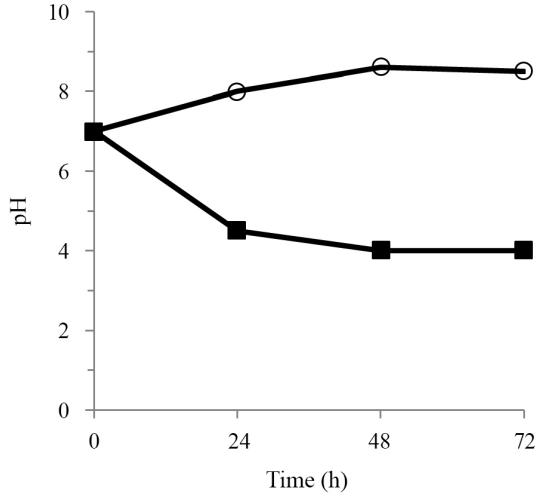

Fig. 5. pH of *Acinetobacter* sp. B W (■) and cured strain B-W (○) during growth in the modified PGI medium at 28°C.

Effect of plasmid loss on antibiotic resistance and MICs of *Acinetobacter* sp. B-W

A comparison of the antibiotic resistant traits between strain B-W, cured strain and transformant *E. coli* DH5α are shown in Table 1. Strain B-W and transformant *E. coli* DH5α exhibited resistance to ampicillin, actinomycin D, bacitracin, lincomycin, and vancomycin, but cured strain B-W was sensitive to those antibiotics. Based on the above fact, it can be concluded that the resistance to antibiotics by the strain B-W was mediated by plasmid of approximately 20 kb. Earlier it was noted that EtBr curing led to disappearance of resistance to extended spectrum of β-lactams (ESBL) with the concurrent loss of plasmids from *A. baumannii* suggesting that ESBL determinants were plasmid-borne (Kenchappa and Sreenivasmurthy, 2003). The MIC distributions observed for antibiotics are summarized in Table 2. Plasmid curing of strain B-W resulted in drastic reduction of MIC of several antibiotics (ampicillin, actinomycin D, bacitracin, lincomycin, and vancomycin). MICs of transformant *E. coli* DH5α were increased to the level of wild strain B-W. These results showed that antibiotic resistant gene were plasmid-borne. It has been reported that plasmid-borne bla_{OXA-51} bearing isolates had higher MICs to carbapenems than those bearing bla_{OXA-51} only on chromosomes (Saranathan *et al*., 2014). This could be due to increased gene dosage provided by the higher copy number of associated plasmids.

Table 1. Antibiotic susceptibility test by disc diffusion method

Strains	Amp	Act-D	Bac	Lin	Van
B-W	R	R	R	R	R
Cured B-W	S	S	S	S	S
Transformant *E. coli* DH5α	R	R	R	R	R

R, resistant; S, sensitive; Amp, ampicillin; Act-D, actinomycin D; Bac, bacitracin; Lin, lincomycin; Van, vancomycin.

Table 2. Determination of minimal inhibitory concentration (MIC)

Strain \ Ant	MIC (μg/ml)				
	Amp	Act-D	Bac	Lin	Van
B-W	800	512	256	512	400
Cured B-W	12.5	2.5	1.2	6.4	3.2
Transformant *E. coli* DH5α	800	512	256	512	400

Ant, Antibiotics; Amp, ampicillin; Act-D, actinomycin D; Bac, bacitracin; Lin, lincomycin; Van, vancomycin

적 요

시드로포어인 2, 3-dihydroxybenzoic acid (DHB)를 생산하는 *Acinetobacter* sp. B-W의 플라스미드를 분석한 결과, 20 kb 크기의 플라스미드를 함유하였다. 배양 온도 43°C가 플라스미드가 제거된 돌연변이체 생산에 효과적이었다. 이 돌연변이체는 2,3-DHB 생산 능력을 소실하였으며, chrome azurol S (CAS) 아가 배지에서 시드로포어 생산이 검출되지 않았다. 포도당과 황산 망간을 함유한 배지에서 28°C로 3일간 배양한 B-W 원 균주와 돌연변이체의 배양 상등액의 pH는 각각 4.5와 8.5로 나타났다. 돌연변이체에서는 ampicillin, actinomycin D, bacitracin, lincomycin과 vancomycin 같은 항생제에 대한 저항성이 사라졌으며, 이러한 항생제에 대한 최소 억제 농도 (MIC)가 급격하게 감소하였다. B-W 균주에서 분리한 플라스미드로 대장균을 형질전환시킨 결과, 원 균주와 같은 크기의 플라스미드가 이 형질전환 대장균에서 발견되었다. 플라스미드가 제거된 돌연변이체에서는 플라스미드가 발견되지 않았다. 20 kb 크기의 플라스미드에 2,3-DHB 생산 유전자와 여러 항생제 저항성 유전자가 자리잡고 있는 것으로 추정된다.

Acknowledgements

This work was supported by Soonchunhyang University Research Fund.

References

Arnow, L.E. 1937. Colorimetric determination of the components of 3, 4-dihydroxyphenylalanine-tyrosine mixtures. *J. Biol. Chem*. **18**, 531–537.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol*. **45**, 493–496.

Chen, Q., Actis, L.A., Tolmasky, M.E., and Crosa, J.H. 1994. Chromosome-mediated 2, 3-dihydroxybenzoic acid is a precursor in the biosynthesis of the plasmid mediated siderophore anguibactin in *Vibrio anguillarum*. *J. Bacteriol*. **176**, 4226–4234.

Clinical and Laboratory Standards Institute. 2010. Performance Standards for Antimicrobial Susceptibility Testing: Twentieth Informational Supplement M100-S20. CLSI, Wayne, PA, USA.

Driss, F., Tounsi, S., and Jaoua, S. 2011. Relationship between plasmid loss and gene expression in *Bacillus thuringiensis*. *Curr. Microbiol*. **62**, 1287–1293.

Elias, H.M., Qader, M.K., and Salih, W.M. 2013. Determination of plasmid DNA role in multidrug resistant *Pseudomonas aeruginosa* clinical isolates. *Int. J. Microbiol. Immunol. Res.* **1**, 80–86.

González, J.M., Dulmage, H.T., and Carlton, B.C. 1981. Correlation between specific plasmids and delta-endotoxin production in *Bacillus thuringiensis*. *Plasmid* **5**, 351–365.

Jalal, M.A.F., Hossain, M.B., Helm, D., Sanders, J., Acis, L.A., and Crosa, J.H. 1989. Structure of anguibactin, a uique plasmid related bacterial siderophore from the fish pathogen *Vibrio anguillarum*. *J. Am. Chem. Soc*. **111**, 292–296.

Kenchappa, P. and Sreenivasmurthy, B. 2003. Simplified panel of assimilation tests for identification of *Acinetobacter* species. *Indian J. Pathol. Microbiol*. **246**, 700–706.

Kim, K.J., Lee, J.H., and Yang, Y.J. 2015. Temperature dependent 2, 3-dihydroxybenzoic acid production in *Acinetobacter* sp. B-W. *Korean J. Microbiol*. **51**, 249–255.

Maniatis, T., Fritsch, E.F., and Sambrook, J. 1989. Molecular cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Meyers, J.A., Sanchez, D., Elwell, L.P., and Falkow, S. 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. *J. Bacteriol*. **123**, 1529–1537.

Miethke, M. and Marahiel, M.A. 2007. Siderophore-based iron acquisition and pathogen control. *Microbiol. Mol. Biol. Rev*. **71**, 413–451.

Saranathan, R., Sudhakar, P., Karthika, R.U., Singh, S.K., Shashikala, P., Kanungo, R., and Prashanth, K. 2014. Multiple drug resistant carbapenemases producing *Acinetobacter baumannii* isolates harbours multiple R-plasmids. *Indian J. Med. Res*. **140**, 262–270.

Schwyn, B. and Neiland, J.B. 1987. Universal chemical assay for detection and determination of siderophores. *Anal. Biochem.*

160, 47–56.

Steers, E., Foltz, E.L., Graves, B.S., and Riden, J. 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. *Antibiot. Chemother. (Northfield)* **9**, 307–311.

Wandersman, C. and Delepelaire, P. 2004. Bacterial iron sources: from siderophores to hemophores. *Annu. Rev. Microbiol.* **58**, 611–647.

Wencewicz, T., Möllmann, U., Long, T., and Miller, M. 2009. Is drug release necessary for antimicrobial activity of siderophore-drug conjugates? Syntheses and biological studies of the naturally occurring salmycin "Trojan Horse" antibiotics and synthetic desferridanoxamine antibiotic conjugates. *BioMetals* **22**, 633–648.

Young, I.G. and Gibson, F. 1969. Regulation of the enzymes involved in the biosynthesis of 2, 3-dihydroxybenzoic acid in *Aerobacter aerogenes* and *E. coli*. *Biochim. Biophys. Acta*. **177**, 401–411.

Zhang, R., Pan, L., and Zhao, Z. 2012. High incidence of plasmids in marine *Vibrio* species isolated from Mai Po Nature Reserve of Hong Kong. *Ecotoxicology* **21**, 1661–1668.

Zurkowski, W. and Lorkiewcz, Z. 1978. Effective method for the isolation of non-nodulation mutants of *Rhizobium trifolii*. *Genet. Res*. **32**, 311–314.