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Effect of plasmid curing on the production of siderophore from glutamic acid as both carbon and nitrogen sole sources in *Acinetobacter* sp. B-W

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글루탐산을 유일한 탄소 원과 질소 원으로 이용하는 *Acinetobacter* sp. B-W의 글루탐산으로부터의 시드로포어 생산에 미치는 플라스미드 제거 효과

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Effect of plasmid curing of *Acinetobacter* sp. B-W on the production of siderophore from glutamic acid as both carbon and nitrogen sole sources was investigated. Plasmid cured mutant of strain B-W lost the ability to produce siderophore from glutamic acid at 28°C. Transformant *E. coli* DH5α harboring 20 kb plasmid, that was isolated from wild type of strain B-W produced siderophore from glutamic acid as both carbon and nitrogen sole sources at 28°C, but, not at 36°C. Production of siderophore from glutamic acid by transformant *E. coli* DH5α was completely inhibited by 10 μM $FeCl_3$. In previous report, catechol nature of siderophore produced from glutamic acid by strain B-W was detected by Arnow test. The siderophore produced from glutamic acid by transformant *E. coli* DH5α was also catechol type. Rf value of siderophore produced from transformant *E. coli* DH5α grown in medium glutamic acid as both carbon and nitrogen sole sources at 28°C was 0.32 in butanol-acetic acid-water (12:3:5) as developing solvent. Rf value of the siderophore was the same with that of wild type of strain B-W. Thus a single plasmid of 20 kb seemed to be involved in the production of siderophore from glutamic acid.

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Siderophores are compounds secreted under low iron stress, that act as a specific ferric iron chelate agents (Wandersman and Delepelaire, 2004). Siderophores involve hydroxamate, catecholate, phenolate, *α*- hydroxycarboxylate, or mixed-function groups capable to form coordination complexes with very high affinity and selectivity for ferric ions (Hider and Kong, 2010). In our previous report (Kim *et al*., 2015), siderophore produced from *Acinetobacter* sp. B-W grown in medium containing glucose as a carbon source and sodium glutamate as a nitrogen source was identified as a 2, 3-dihydroxybenzoic acid (DHB). But, in the medium containing glutamic acid as both carbon and nitrogen sole sources, strain B-W produced siderophore different from 2, 3-DHB (Kim *et al.*, 2017). Glutamate is known as major amino donor for most anabolic enzymatic reactions and yet also functions as a key intermediate in carbon metabolism (Sonenshein, 2007; Gunka and Commichau, 2012). Curing is

the artificial way used by scientists to make microorganisms lose genetic material, mainly plasmids (Gonza´lez *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. We have reported that a single plasmid of 20 kb of strain B-W seemed to be involved in 2, 3-DHB production (Kim *et al*., 2016). In this study, we have investigated the effect of plasmid curing on the production of siderophore from glutamic acid as both carbon and nitrogen sole sources in strain B-W.

Materials and Methods

Microorganisms, media, and culture conditions

Isolation and characterization of *Acinetobacter* sp. B-W was described in our previous report (Kim *et al*., 2015). *Acinetobacter* sp. B-W was isolated from soil in Cebu city of Philippines. *Acinetobacter* species are Gram-negative bacteria belonging to the wider class of Gammaproteobacteria, and non-motile, oxidase-negative, catalase positive and occur in pairs under magnification. *Acinetobacter* sp. B-W was maintained on a semi synthetic minimal medium: 5 g glucose, 0.05 g $(NH_4)_2SO_4$, 0.05 g yeast extract, 0.2 g KH_2PO_4, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.005 g $FeSO_4$, and 20 g agar per 1 L (pH 7.0). For glutamic acid as both carbon and nitrogen sole sources, following compositions were used as a siderophore production medium at 28°C; 30 g sodium glutamate, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 g K_2HPO_4, 0.002 g $MnSO_4$ per 1 L (pH 7.0).

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

Plasmid curing was done as described in our previous report (Kim *et al*., 2016) 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 43°C and transferred at weekly intervals. Growth at 43°C was repeated for several times in fresh medium along with occasional monitoring of the plasmid profile in agarose gel. Loss of siderophore production of the cured mutant of strain B-W was checked in the glutamic acid as both carbon and nitrogen sole sources containing medium at 28°C.

Siderophore detection

The Chrome Azurol S (CAS) plates (Schwyn and Neiland, 1987) was 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 catechol type siderophore of *Acinetobacter* sp. B-W grown in the glutamic acid as both carbon and nitrogen sole sources containing medium at 28°C was examined by the Arnow reaction (Arnow, 1937).

Transformant *E. coli* DH 5α

Transformant *E. coli* DH5α containing 20 kb plasmid that purified from wild type strain B-W was used as described in our previous report (Kim *et al*., 2016). Transformation of *E. coli* DH5 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 pBR32 was made on NA containing ampicillin (100 μg/ml). Production of siderophore was tested in the supernatant of transformant *E. coli* DH5α grown in the glutamic acid as both carbon and nitrogen sole sources containing medium at 28°C.

Isolation and purification of siderophore

Isolation and purification of siderophore of strain B-W and transformant *E. coli* DH5α were performed as described in our previous report (Kim *et al*., 2017). Bacterial cultures grown in 600 ml of medium containing glutamic acid as both carbon and nitrogen sources for 48 h at 28°C were harvested by centrifugation at 8,000 rpm for 10 min. Iron-free siderophores were obtained by the following method (Payne, 1994; Milagres *et al*., 1999). Culture supernatants were extracted three times with equal volumes of butanol. The concentrated organic extracts were dissolved in 5 ml of methanol. Partial purification of the siderophores was achieved by the fractionation of the organic extracts on a Sephadex LH-20 (Pharmacia) column in the methanol elution. The eluting solutions were purified with

Chelex-100 to remove the iron. Arnow reaction positive fractions were pooled and concentrated 10-fold by evaporation.

TLC

Thin-layer chromatography (TLC) was performed on 0.25-mm-thick silica gel 60 F254 in the following solvent systems as described in our previous report (Kim *et al*., 2015); Benzene-acetic acid-water (120:70:3), Butanol-acetic acid-water (12:3:5), Butanol-pyridine-water (14:3:3), chloroform-methanol (2:1), Toluene-1,4-dioxane-acetic acid (45:10:2). The plates were examined under UV light or sprayed with 0.12 N $FeCl_3$ in 0.1 N HCl to detect iron-binding compounds (O'Brien *et al*., 1970). To detect catechol-type compounds, they were sprayed with either the reagents of the Arnow assay or 1% ferric ammonium citrate and then with potassium ferricyanide (Rogers, 1973).

Results and Discussion

Effect of plasmid loss on growth and siderophore production of *Acinetobacter* sp. B-W

Isolated plasmid from *Acinetobacter* sp. B-W was analyzed by agarose gel electrophoresis.

The size of plasmid was 20 kb (Fig. 1). The strain B-W was subjected to plasmid curing (Zurkowski and Lorkiewcz, 1978) at 43°C. Plasmid curing affected the growth rate of *Acinetobacter* sp. B-W. Growth rate of cured strain B-W in medium containing glutamic acid as both carbon and nitrogen sole sources was approximately 82% of wild type strain B-W (Fig. 2). Significant difference was observed in the siderophore production of plasmid cured strain B-W when compared to the parental one. Production of siderophore of strain B-W was totally lost by curing (Fig. 3). Catechol type sideropore was assayed by Arnow test (Arnow, 1937) at 510 nm. Production of siderophore was detected on a CAS agar plate. Orange halos of supernatant from strain B-W and transformant *E. coli* DH5α were 18–20 mm, but

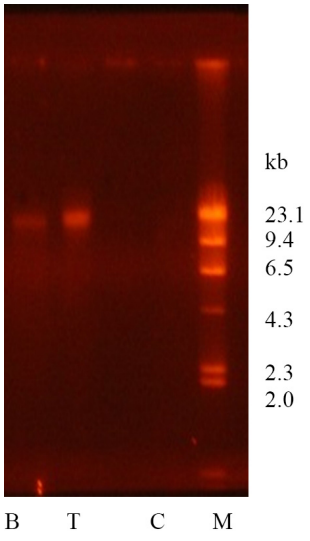

Fig. 1. Agarose gel electrophoresis of plasmid DNA from *Acinetobacter* sp. B-W (B), transformant (T) and cured strain B-W (C), M: DNA standard marker.

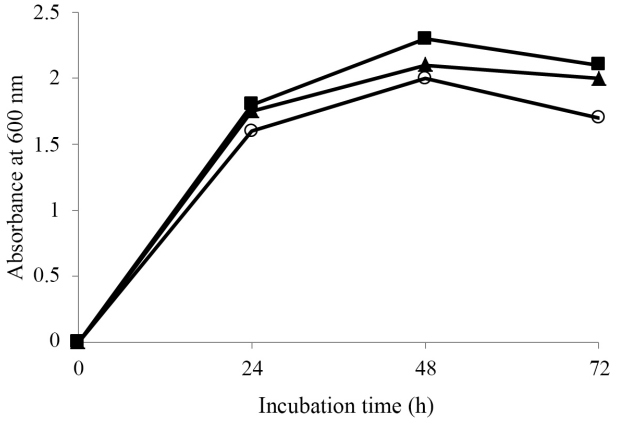

Fig. 2. Growth curve of *Acinetobacter* sp. B-W (■), cured strain B-W (○), and transformant *E. coli* DH5α (▲) grown in medium containing 3% glutamic acid as both carbon and nitrogen sources at 28°C.

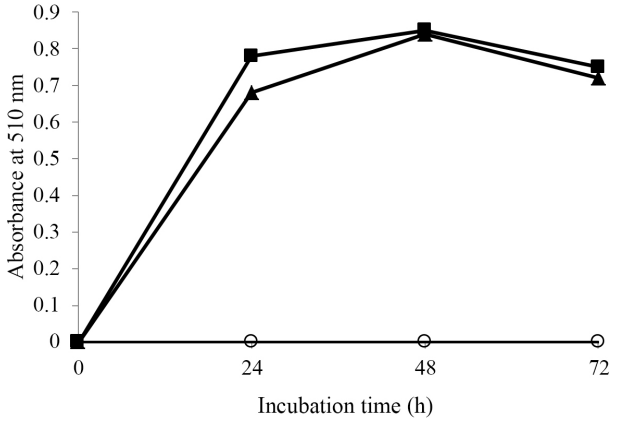

Fig. 3. Production of siderophore by *Acinetobacter* sp. B-W (■), cured strain B-W (○) and transformant *E. coli* DH5α (▲) during growth. Cells were grown in medium containing glutamic acid as both carbon and nitrogen sources at 28°C for the determination of siderophore by Arnow test.

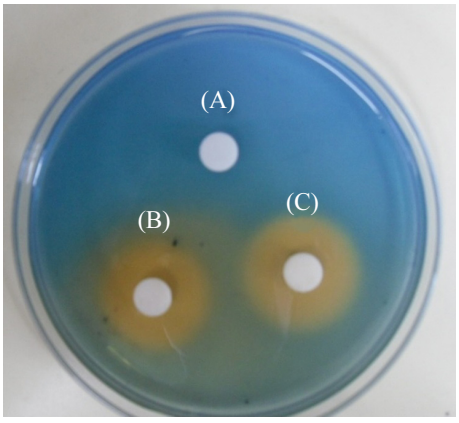

Fig. 4. CAS agar assay. The CAS agar assay was performed on CAS plate with paper discs those were coated with followings; (A) supernatant of cured *Acinetobacter* sp. B-W grown in medium containing glutamic acid as both carbon and nitrogen sources at 28°C for 24 h, (B) supernatant of *Acinetobacter* sp. B-W grown for 24 h , (C) supernatant of transformant *E. coli* DH5α grown for 24 h.

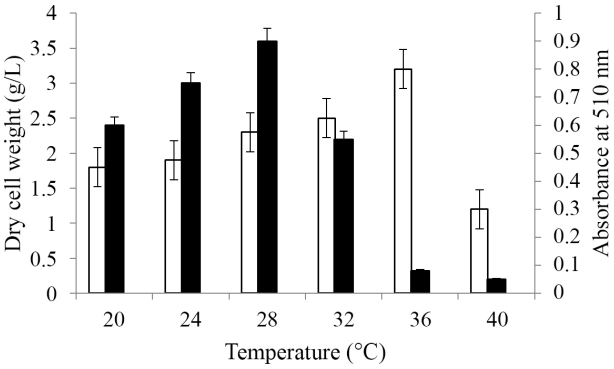

Fig. 5. Growth and siderophore production by transformant *E. coli* DH5α grown in medium containing glutamic acid as both carbon and nitrogen sources at different temperatures for 48 h. (□) cell dry weight, (■) siderophore production. Error bars correspond to standard deviations from triplicate replicas.

that of cured strain B-W was 0 mm (Fig. 4). Disappearance of siderophore production with concurrent loss of plasmid from strain B-W suggested that siderophore biosynthetic genes were plasmid-borne. It was confirmed from the result that siderophore production was restored in the transformant *E. coli* DH5α (Figs. 3 and 4). Plasmid mediated siderophore, anguibactin production was reported in *Vibrio anguillarum* (Chen *et al*., 1994). But, it is known that catecholate siderophore, anthrachelin genes are chromosomal in *Bacillus anthracis* (Garner *et al*., 2004).

Effect of temperature on the growth of cured strain B-W and transformant *E. coli* DH5α

The influence of growth temperature on siderophore production of transformant *E. coli* DH5α was studied by growing cultures in 3% glutamic acid as both carbon and nitrogen sources at 20°C, 24°C, 28°C, 32°C, 36°C, or 40°C (Fig. 5). *E. coli* DH5α wild type didn't grow in medium containing 3% glutamic acid as both carbon and nitrogen sources. Optimal growth temperature of strain transformant *E. coli* DH5α is around 36°C. The growth rate steadily increases with temperature from 20 to 36°C. Above 40°C, the growth rate decreases drastically. Siderophore production was increased with temperature from 20°C to 28°C and then decreased from 32°C to 40°C. Transformant *E. coli* DH5α failed siderophore production at 36°C at detectable levels and it could indicate that siderophore synthesis of transformant *E. coli* DH5α is temperature sensitive. Cured strain B-W produced siderophore neither at 28°C nor 36°C Temperature regulated siderophore production has been identified in several bacterial genera (Garibaldi, 1972; Cogswell and Weinberg, 1980; Worsham and Konisky, 1984) and this type regulation in which siderophore production is reduced at elevated temperatures has been directly associated with virulence in *Pseudomonas aeruginosa* (Meyer *et al*., 1996).

Effect of iron concentration on the growth and production of siderophore of transformant *E. coli* DH5α

E. coli DH5α was studied in medium containing 3% glutamic acid both as carbon and nitrogen source with varying amounts of iron. Transformant *E. coli* DH5α was sensitive to the increase of iron concentration in the medium (Fig. 6). In transformant *E. coli* DH5α, a concentration of iron less than 2 μM was needed for siderophore production. Higher concentration of iron considerably decreased the siderophore production. At a concentration of 10 μM in the medium, the siderophore production of transformant *E. coli* DH5α was completely inhibited. As shown in Fig. 7, although iron supplementation in liquid cultures accelerated growth of transformant *E. coli* DH5 α, differential levels of iron availability did not greatly influence maximum growth, which may be explained by successful bacterial adaptation to different culture conditions regarding iron availability. In general, the growth curves were not much

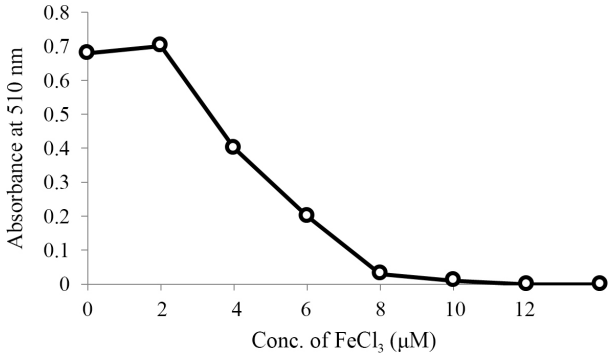

Fig. 6. Effect of ferric ion concentrations on the production of siderophore by transformant *E. coli* DH5α grown in medium containing glutamic acid as both carbon and nitrogen sources at 28°C. The siderophore concentration was estimated by Arnow test.

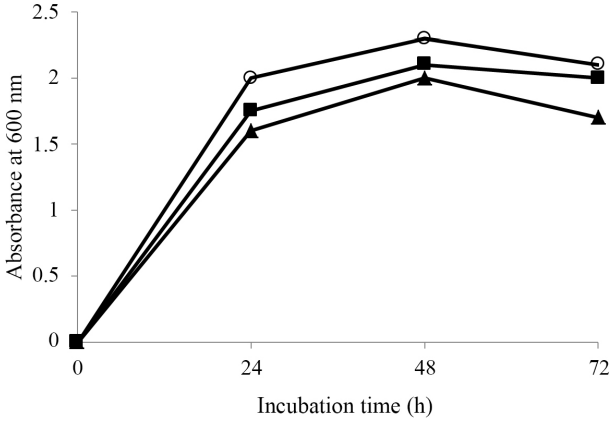

Fig. 7. Growth curve of transformant *E. coli* DH5α grown in different ferric ion concentrations. (▲) 0, (■) 2 μM, (○) 10 μM.

different depending on the iron concentration in the medium. Up to 24 h, the growth rate was fast for all three culture conditions (with $FeCl_3$ 0, 2, and 10 μM). After this period, slight increase in growth and then no significant increase in the growth was observed. Our experimental data suggest that more siderophores were produced when transformant *E. coli* DH5α grew slowly (under iron deficiency) than when it grew rapidly (under iron supplementation). This fact agrees with the statement made by Neilands (1984) that high iron concentration in the medium generally results in excellent microorganism growth but only modest yields of siderophore.

TLC

As shown in Table 1, Rf values of siderophore from transformant *E. coli* DH5α grown in the medium containing glutamic acid as carbon and nitrogen sources at 28°C were different from that of 2, 3-dihydroxybenzoic acid (DHB). Rf value of siderophore from transformant *E. coli* DH5α was 0.32, while 2, 3-DHB was 0.84 in butanol-acetic acid-water (12:3:5) as developing solvent (Fig. 8) This result showed that transformant *E. coli* DH5α produced ferric ion chelating molecules different from 2, 3-DHB.

Table 1. Rf values of $FeCl_3$ reacting components produced from strain B-W and from transformant *E. coli* DH5α grown in medium containing L-glutamic acid as both carbon and nitrogen sole sources

Rf value		
Solvent system	B-W	Transformant *E. coli* DH5α
Benzene-acetic acid-water (120:70:3)	0.54	0.53
Butanol-acetic acid-water (12:3:5)	0.32	0.32
Butanol-pyridine-water (14:3:3)	0.58	0.57
Chloroform-methanol (2:1)	0.34	0.34
Toluene-1,4-dioxane-acetic acid (45:10:2)	0.44	0.44

Thin-layer chromatography (TLC) was performed on 0.25-mm-thick silica gel 60 F254.
Detection reagent: 1% ferric ammonium citrate and then with potassium ferricyanide.

(A) (B) (C)

Fig. 8. TLC analysis of purified siderophore. (A) siderophore produced from *Acinetobacter* sp. B-W grown in medium containing glutamic acid as both carbon and nitrogen sources at 28°C (B) 0.5% 2, 3-Dihydroxybenzoic acid, (C) siderophore produced from transformant *E. coli* DH5α. TLC F254 silica gel plate in combination with a mixture of butanol : acetic acid : water (12 : 3: 5, v/v/v) as mobile phase was used. Plate was sprayed with 1% ferric ammonium citrate and then with potassium ferricyanide.

적 요

플라스미드가 제거된 *Acinetobacter* sp. B-W 균주의 돌연변이체를 글루탐산을 유일한 탄소 원과 질소원으로 함유한 배지에 28°C에서 배양한 결과 글루탐산으로부터의 시드로포어 생산이 억제되었다. B-W 원 균주의 20 kb 플라스미드를 가진 형질 전환체 대장균 DH5α는 같은 조건의 배지에서 시드로포어를 생산하는 것으로 조사되었다. 그러나 36°C에서는 형질 전환체 대장균 DH5α의 시드로포어 생산이 강하게 억제되었으며, 돌연변이체 B-W 균주는 28°C에서와 마찬가지로 36°C에서도 시드로포어를 생산하지 못하였다. 형질 전환체로부터 생산된 시드로포어의 종류는 원 균주 B-W와 마찬가지로 Arnow 시험 결과 카테콜 형으로 조사되었으며, 10 μM $FeCl_3$를 첨가한 배지에서는 시드로포어 생산이 완전히 억제되었다. 형질 전환체로부터 생산된 시드로포어의 TLC상에서의 Rf값은 butanol-acetic acid-water (12:3:5) 용매상에서 0.32로 원 균주 B-W에서 생산된 시드로포어와 같았다. 위와 같은 실험 결과로 글루탐산으로부터 생산된 시드로포어의 생합성에 관여하는 유전자들이 20 kb 플라스미드 상에 있는 것으로 추정되었다.

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