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Article

Production of siderophore from L-glutamic acid as both carbon and nitrogen sole sources in *Acinetobacter* sp. B-W

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글루탐산을 유일한 탄소원과 질소원으로 이용하는 *Acinetobacter* sp. B-W의 시드로포어 생산

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Catechol type siderophore different from 2, 3-dihydroxybenzoic acid (DHB) was produced from *Acinetobacter* sp. B-W grown in medium containing L-glutamic acid as both carbon and nitrogen sole sources at 28°C. Optimal concentration of glutamic acid for siderophore production was 3% and production of siderophore was decreased above 3% glutamic acid. In previous report, siderophore, 2, 3-DHB was produced from strain B-W grown in medium containing glucose as carbon source and glutamic acid as nitrogen source. Rf value of siderophore produced from strain B-W grown in medium glutamic acid as both carbon and nitrogen sole sources at 28°C was 0.32, while 2, 3-DHB was 0.84 in butanol-acetic acid-water (12:3:5) as developing solvent. Antioxidative activity of 2, 3-DHB was not detected in that siderophore produced from glutamic acid. Catechol nature of siderophore was detected by Arnow test. Although in iron-limited media optimal cell growth was identified at 36°C, significant quantities of siderophore were produced only at 28°C. Biosynthesis of siderophore was strongly inhibited by growth at 36°C. Production of siderophore was completely inhibited by 10 μM $FeCl_3$.

Keywords: *Acinetobacter* sp. B-W, L-glutamic acid, siderophore, sole carbon and nitrogen sources

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Iron is an essential micronutrient for almost all living organisms. Although iron is an abundant element, its bio-availability in aerobic environments is limited because of the insolubility of ferric iron. 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). Siderophores are low molecular weight compounds (400~1,000 Da) with high affinity for ferric ion (Raymond and Dertz, 2004; Skaar, 2010). Broadly, siderophores can be classified into three categories depending upon the moiety that donates oxygen ligands for Fe^{+3} coordination: a) catecholates (or phenolates), b) hydroxymates (or carbooxylate), and c) the mixed types (Miethke and Marahiel, 2007; Wencewicz *et al*., 2009). Many bacteria, including *Pseudomonas* sp., *Azotobacter* sp., *Rhizobium* sp., *Bacillus* sp., etc. are able to excrete catechol type siderophores (Hoefte, 1993; Carrillo-Castaneda *et al*., 2000). In our previous report (Kim *et al*., 2015), catechol type siderophore produced from *Acinetobacter* sp. B-W grown in the modified PGI medium containing glucose and glutamic acid was identified as a 2, 3-dihydroxybenzoic acid (DHB). In the modified PGI medium

glutamic acid used as nitrogen source. Nitrogen metabolism is strongly interconnected with the energy metabolism of the cell because of the dual dependency on ATP, NAD, or NADPH as co-factors (Magasanik, 2003). In this study, we have tested the ability of strain B-W to utilize glutamic acid C-catabolically and to produce siderophore. Properties of siderophore produced from glutamic acid are also compared with that of 2, 3-DHB.

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: 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 in 1 L (pH 7.0). The 2, 3-DHB production medium for strain B-W was a chemically defined one (modified PGI medium, pH 7.0) with the following compositions: 5 g glucose, 2.5 g sodium glutamate, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 g K_2HPO_4, 0.002 g $MnSO_4$ in 1 L. For glutamic acid as both carbon and nitrogen sole sources, following compositions were used as a siderophore production medium; 30 g sodium glutamate, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 g K_2HPO_4, 0.002 g $MnSO_4$ in 1 L. The effect of iron concentration of iron on the siderophore production was tested by adding $FeCl_3 \cdot 6H_2O$ to the medium.

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. Hydroxamate and catechol functionality of 10-fold-concentrated siderophore extracts of *Acinetobacter* sp. B-W were examined by the Csaky test (Csaky, 1948; Gillam *et al*., 1981) and the Arnow reaction (Arnow, 1937), respectively. In these assays, hydroxylamine and 2, 3-dihydroxybenzoic acid, respectively, were used as the standards.

Determination of concentration of glutamic acid

The glutamate content of the cultures was determined colorimetrically at 570 nm as described by Yokoyama and Hiramatsu (2003). Ninhydrin reagent is prepared by dissolving 0.5 g of ninhydrin and 15 mg ascorbic acid into methyl cellosolve. To 1 ml of sample, 2 ml of 0.5 M sodium citrate buffer (pH 5.0), and 1 ml of ninhydrin solution containing ascorbic acid were added, and the mixture was heated at 100°C for 15 min and immediately cooled in an ice bath (0°C). After the addition of 1 ml of 60% ethanol, the absorbance was measured at 570 nm. Sodium glutamate monohydrate was used as the standard.

DPPH (α,α–Dihenyl–β–picrylhydrazyl) radical scavenging activity

Antioxidant activity was measured using DPPH radical scavenging assay method. A 0.5 ml sample was mixed with 0.25 ml of an ethanolic 0.5 mM DPPH solution and 0.5 ml of 100 mM acetate buffer (pH 5.5). The tubes were mixed for 15 sec, and after standing for 30 min, the absorbance of the mixture was measured at 517 nm (Abe *et al*., 1998).
The percent of DPPH decoloration of the sample was calculated according to the formular,

% decoloration = (1- Abs sample / Abs control) × 100.

where Abs sample and Abs control were the absorbance of the sample and the control, respectively.

Effect of glutamate concentration on the growth and siderophore production

Effect of glutamate concentrations (0.25~5%) on the growth and siderophore production of strain B-W grown in the medium containing glutamic acid as both carbon and nitrogen sources was investigated.

Isolation and purification of siderophore

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: 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 either the reagents of the Arnow assay or 1% ferric ammonium citrate and then with potassium ferricyanide (Rogers, 1973).

Results and Discussion

Effect of concentration of glutamic acid on the growth and siderophore production

As shown in Fig. 1, growth rate of strain B-W was gradually increased in the glutamate concentration from 0.25 to 2%, above 2% it was slightly increased. In the concentration of glutamate from 0.25 to 0.5%, glutamate was nearly exhausted after 48 h (data not shown) and accordingly the growth ceased. Production of siderophore was increased between 0.25 and 3.0% of glutamate concentration (Fig. 2). The maximum value of glutamate as an effective concentration for siderophore production was reached at 3%. The maximized siderophore production was decreased beyond 3% of glutamate. According to the results in Fig. 2, the concentration of 3% was selected for further test of siderophore production of strain B-W growing on glutamic acid as sole carbon and nitrogen sources. 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). Our results suggest that glutamate serves as a good carbon source to facilitate growth in media lacking a preferred carbon source such as glucose.

Effect of temperature on the growth and siderophore production

The influence of growth temperature on siderophore production of strain B-W was studied by growing cultures in 3% glutamic acid as both carbon and nitrogen sources at 20, 24, 28, 32, 36, or 40°C (Fig. 3). Optimal growth temperature of strain B-W 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 to 28°C and then decreased from 32 to 40°C. Strain B-W failed siderophore production at 36°C at

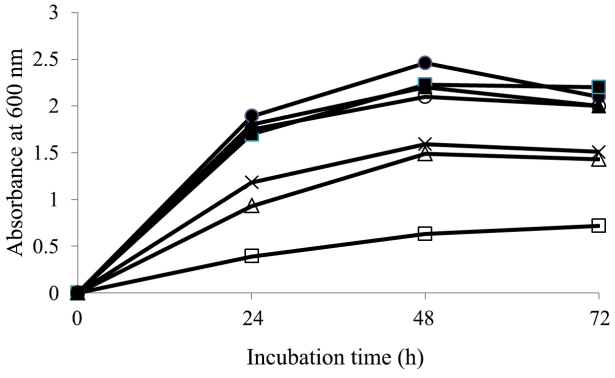


Fig. 1. Effect of concentrations of glutamic acid on the growth of *Acinetobacter* sp. B-W grown in medium containing glutamic acid as both carbon and nitrogen sources at 28°C (Glutamic acid: □, 0.25%; △, 0.5%; X, 1 %; ○, 2%; ■, 3%; ●, 4%; ▲, 5%).

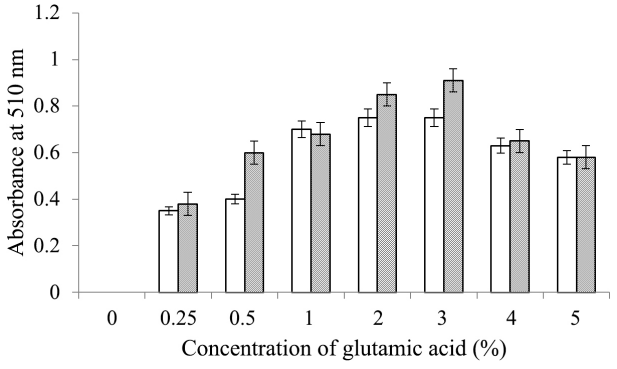


Fig. 2. Effect of concentrations of glutamic acid on the production of siderophore from *Acinetobacter* sp. B-W. Production of siderophore was measured by Arnow test.
□, 24 h; ▨, 48 h. Error bars correspond to standard deviations from triplicate replicas.

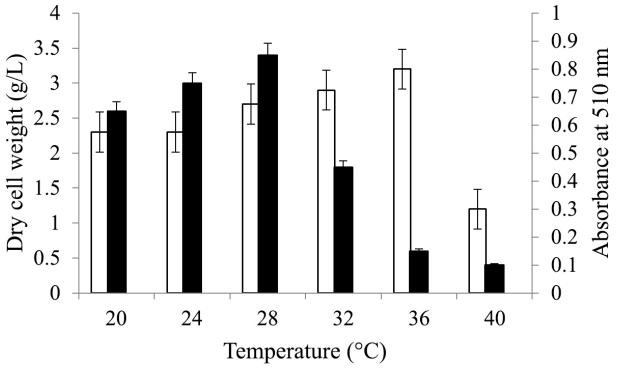


Fig. 3. Growth and siderophore production by *Acinetobacter* sp. B-W grown in medium containing glutamic acid as both carbon and nitrogen sources at different temperatures for 48 h. □, dry weight; ■, siderophore production. Error bars correspond to standard deviations from triplicate replicas.

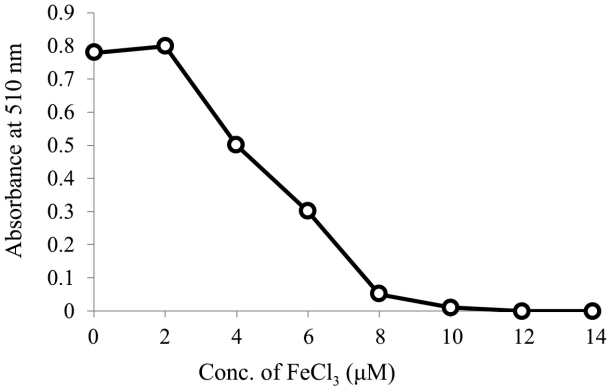


Fig. 4. Effect of iron concentrations on the production of siderophore by *Acinetobacter* sp. B-W grown in medium containing 3% glutamic acid as carbon and nitrogen sources at 28°C for 48 h. The siderophore concentration was estimated by Arnow test.

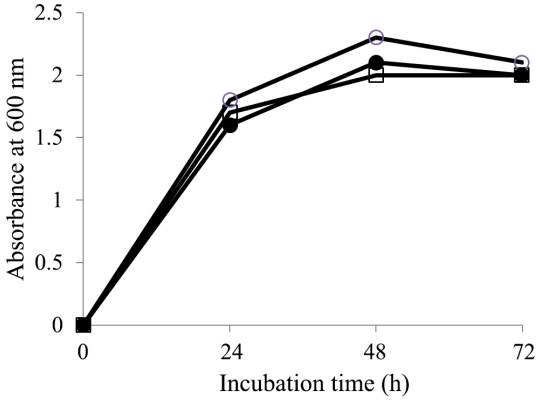


Fig. 5. Growth curve of *Acinetobacter* sp. B-W grown in medium containing 3% glutamic acid as both carbon and nitrogen sources under different iron concentrations at 28°C. □, 0; ●, 2 μM; ○, 10 μM.

detectable levels and it could indicate that siderophore synthesis of strain B-W is temperature sensitive. 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). High growth levels in the absence of siderophore in strain B-W at 36°C indicate that stain B-W may possess an alternative non-siderophore based iron assimilation system. It has been identified up to five iron assimilation mechanisms in *E. coli* (Earhart, 1996).

Effect of iron concentration on the production of siderophore and growth rate

The effect of iron concentrations on the siderophore production by *Acinetobacter* sp. B-W was studied in medium containing 3% glutamic acid both as carbon and nitrogen source with varying amounts of iron. Strain B-W was sensitive to the increase of iron concentraion in the medium (Fig. 4). In strain B-W, 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 B-W was completely inhibited. As shown in Fig. 5, although iron supplementation in liquid cultures accelerated growth of strain B-W, 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 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 strain B-W 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.

Characterization of siderophore

Functionality of siderophore produced from strain B-W grown in the medium containing 3% glutamic acid both as carbon and nitrogen source was determined to catechol type by Arrow assay (Arnow, 1937). Csaky test (Csaky, 1948), that is useful for the detection of hydroxamate type siderophore, gave negative results. Many high-affinity ferric ion chelating molecules contain catecholate that are part of the iron-binding site (Loehr, 1986; Jalal *et al*., 1989; Walsh *et al*., 1990). Antioxidative activity of siderophore from strain B-W was negative result, while 2, 3-DHB was shown as a antioxidative compound (Table 1). Therefore, we assumed that strain B-W produced antioxidative siderophore, 2, 3-DHB in the presence of glucose, while in the presence of glutamic acid as carbon and nitrogen sources it produced different siderophore from 2, 3-DHB. Further work is required to determine the structure of siderophore produced from strain B-W grown in medium containing glutamic acid as sole carbon and nitrogen sources.

Table 1. Antioxidative activity of siderophore produced from *Acinetobacter* sp. B-W

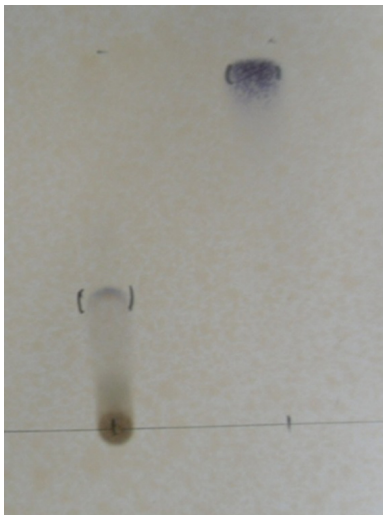
Sample	DPPH radical scavenging activity (%)
2, 3-Dihydroxybenzoic acid (30 μM)	85
Siderophore from strain B-W*	0
Ascorbic acid (30 μM)	58

* Supernatant of strain B-W grown in medium containing 3% glutamic acid as both carbon and nitrogen sources at 28°C was extracted with butanol and purified by using Sephadex LH-20 column as described in 'Materials and Methods'.

Table 2. Rf values of $FeCl_3$ reacting components produced from *Acinetobacter* sp. B-W grown in medium containing L-glutamic acid as both carbon and nitrogen sole sources

Solvent system	Rf value	
	Sample	2, 3-DHB[a]
Benzene-acetic acid-water (120:70:3)	0.54	0.64
Butanol-acetic acid-water (12:3:5)	0.32	0.82
Butanol-pyridine-water (14:3:3)	0.58	0.54
Chloroform-methanol (2:1)	0.34	0.31
Toluene-1,4-dioxane-acetic acid (45:10:2)	0.44	0.38

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]synthetic 2, 3-dihydroxybenzoic acid



(A) (B)

Fig. 6. TLC analysis of purified siderophore. (A) produced from *Acinetobacter* sp. B-W grown in medium containing glutamic acid as both carbon and nitrogen sources at 28°C and (B): 0.5% 2, 3-Dihydroxybenzoic acid. 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.

TLC

As shown in Table 2, Rf values of siderophore from strain B-W 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 strain B-W was 0.32, while 2, 3-DHB was 0.84 in butanol-acetic acid-water (12:3:5) as developing solvent (Fig. 6). This result showed that strain B-W produced ferric ion chelating molecules different from 2, 3-DHB.

적 요

포도당과 글루탐산을 함유한 배지에서 시드로포어인 2, 3-dihydroxybenzoic acid (DHB)를 생산하는 *Acinetobacter* sp. B-W 균주를 글루탐산을 유일한 탄소원과 질소원으로 함유한 배지에 배양한 결과, 상등액에서 2, 3-DHB가 아닌 카테콜 형의 시드로포어를 생산하는 것으로 조사되었다. 글루탐산의 농도는 3%에서 시드로포어 생산이 최고로 나타났으며, 3% 보다 높은 농도에서는 감소하는 것으로 조사되었다. 글루탐산을 유일한 탄소원과 질소원으로 함유한 배지에서 자란 균주 B-W는 배양 온도 28°C에서는 시드로포어를 생산하지만 36°C에서는 생산하지 않았다. 또한 10 μM $FeCl_3$를 첨가한 배지에서는 시

드로포어 생산이 완전히 억제되었다. 글루탐산 배지에서 생산된 균주 B-W의 시드로포어는 TLC 전개 용매 butanol: acetic acid: water =12:3:5에서 Rf치가 0.32로 나타나 Rf치가 0.82인 2, 3-DHB와는 다른 것으로 조사되었으며, 또한 항산화 활성도 없는 것으로 나타나 항산화 활성을 가진 2, 3-DHB와는 다른 시드로포어인 것으로 추정되었다.

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