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Isolation of a Malonate-utilizing *Acinetobacter calcoaceticus* from Soil

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토양으로부터 Malonate를 이용하는 *Acinetobacter calcoaceticus*의 분리

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A bacterium which can utilize malonate as a sole carbon source was isolated from soil. This strain was identified to be *Acinetobacter calcoaceticus* by morphological, cultural, physiological and biochemical examination. When this microorganism was grown on malonate as a sole carbon source, the enzymes, such as malonyl-CoA synthetase, isocitrate lyase and malate synthase were induced. These results suggest that in this microorganism, malonate is also assimilated through the proposed pathway in *Pseudomonas fluorescens:* malonate → malonyl-CoA → acetyl-CoA → glyoxylate cycle.

Biochemical interest in malonate has been still largely directed toward its role as a competitive inhibitor of succinate dehydrogenase, finally terminal respiration. However, the importance of malonate in biological systems has been also gradually recognized since its possible involvement in brain development (Koeppen *et al.*, 1974, Mitzen *et al.*, 1976, Koeppen *et al.*, 1978) and in symbiosis for nitrogen fixation were reported. And biosynthesis and degradation of this dicarboxylate have not been also well studied untill recent years. Recently it has been reported that malonate is assimlated in *Pseudomonas fluorescens* through the metabolic pathway: malonate → malonyl-CoA → acetyl-CoA → glyoxylate cycle. The isolation of the inducible malonyl-CoA synthetase (Kim and Bang, 1985), isocitrate lyase (Jang and Kim, 1982) malate synthase (Chae and Kim, 1984) in *P. fluorscens* grown on malonate as a sole carbon source strongly supported the pathway for the malonate assimilation. That was however the only organism in which malonate assimilation was studied. In this paper we present the isolation of a malonate-utilizing bacteria from soil, the identification of the bacterium as *Acinetobacter calcoaceticus* and in this microorganism the possible existence of metabolic pathway proposed in *P. fluorescens* for malonate assimilation.

MATERIALS AND METHODS

Material

Malonate, ATP, Coenzyme A, DL-isocitrate, acetyl-CoA, and glyoxalate were purchased from Sigma Chem. Co. All other chemicals and reagents were reagent grade. Soil was collected from several area in Seoul.

Isolation of malonate-growing microorganisms

About one gram of soil sample was suspended

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in 100ml of sterile distilled water and shaken for 15min on a rotatory shaker. Then one drop of the supernatant of the suspension was spread on a malonate-agar plate composed of 0.2% malonate, 0.3% NH_4Cl, 0.015 M phosphate buffer (K, Na) pH 6.8, 0.04% $MgSO_4 \cdot 6H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$ and 1.5% noble agar. The plate inoculated with soil microorganisms was incubated at 30°C overnight. After overnight incubation the rapidly growing colonies were collected and reinoculated on the same plate for futher selection.

Identification of microorganism

The cell was identified by the analysis of nutritional requirement, biochemical properties and morphological characteristics by the method of Gerhardt *et al.*(1981) and Gappuccina and Sherman (1983) Utilization of organic compounds as a sole carbon and energy source were conducted in the defined minimal for malonate supplemented with each compound.

Growth condition

The culture medium for *A. calcoaceticus* contained the following composition: 0.6% malonate, 0.3% NH_4Cl, 0.015 M KH_2PO_4-Na_2HPO_4 buffer at pH 6.8, 0.04% $MgSO_4 \cdot 6H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$. The bacteria were grown approximately 10 hr at 30°C with constant mechanical shaking. The cells were harvested in late exponential growth phase by centrifugation and stored in a freezer until needed.

Preparation of cell extracts

A. calcoaceticus cells, kept in a freezer, were thawed and resuspended in 4 volumes of 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM $MgCl_2$, and 1 mM 2-mercaptoethanol. The cells were disrupted by giving ultrasonic waves with a sonicator (Lab-Line Instruments, Inc. U.S.A.) at 4°C. The disrupted cell suspension was centrifuged at 15,000 xg for 20 min and the pellet was discarded.

Enzyme assay

Malonyl-CoA synthetase activity was assayed by measuring the rate of production of malonyl-CoA throught the formation of malonohydroxamate (Jones and Lipmann, 1955). The reaction mixture containing (in micromoles) Tris-HCl buffer, pH 7.4 100; $MgCl_2$, 10; NH_2OH (neutralized with KOH), 200; ATP, 10; CoA 0.2; and enzyme (final volume, 1 ml) were incubated at 30°C for 30 min. The reaction was terminated by the addition of 0.5 ml of 10% trichloroacetic acid. Then 1 ml of 15% $FeCl_3 \cdot 6H_2O$ in 0.66 N HCl was added to the reaction mixture to develop the red-brown color representing the malonohydroxamate-Fe^{3+} complex. The protein precipitate was removed by centrifugation and the color was measured at 540 nm by Shimadzu UV-120 spectrophotpmeter.

Isocitrate lyase was assayed by measuring the amount of glyoxylate formed from trisodium-DL-isocitrate in 10 min at 30°C, glyoxylate was measured by a specific colorimetric method that published by Kornberg (1965). That is, 20 μl of enzyme solution was taken, mixed and preincubated at 30°C for 3 min with 20 mM potassium phosphate buffer (pH 6.1) containing 3 mM $MgCl_2$, and 1 mM 2-mercaptoethanol. The enzymatic reaction was initiated by the addition of 5 mM DL-isocitrate solution followed by through mixing (final volume, 1 ml). After incubation for exactly 10 min at 30°C, 0.1 ml of 1 M oxalic acid was added to the reaction mixture to quench the reaction mixture. Then 0.1ml of concentrated HCl was added and then 0.1ml of 8% (w/v) potassium ferricyanide was added. Ten min after the addition of ferricyanide, the absorbance of the mixture at 520 nm was read.

Malate synthase activity was assayed by the spectrophotometric methods described by Dixon and Konberg (1959) at 30°C with Shimadzu UV 120 spectrophotometer. Protein content was determined by the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Isolation and identification of a malonate-assmilating bacterium

A bacterium which can rapidly utilize malonate as a sole carbon source was isolated from soil. This bacteria was a Gram negative. During exponential growth, the cells were coccobacilli which characteristically occur in pairs and chains, after entry the stationary phase, the cells became

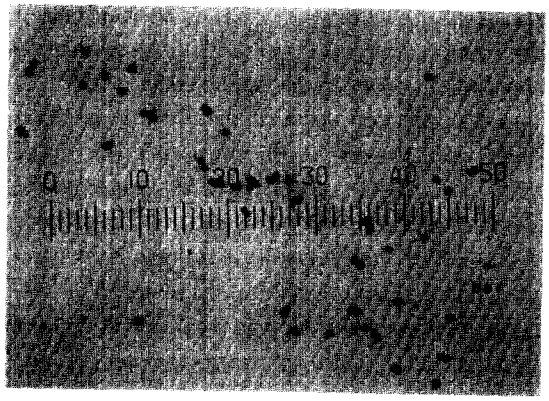

Fig. 1. *Photomicrograph of the isolated malonate-utilizing bacteria. The cells were harvested on the late exponential growth phase and stained with crystal violet. A Nikon Labophot microscope was used for observation. Bar equals 1 μm.*

coccoid, occured single or in pairs, and range in diameter about 1.0 μm (Fig. 1). Other morphological characteristics of the bacteria were summarized in Table 1. This bacteria could grow at between 15°C and 37°C whereas no growth was observed after 5 days incubation at 4°C or 42°C. Optimum growth was achieved at 25-33°C and pH 6.5-8 of the medium. This bacterium was an aerobe. Catalase activity was positive but most of the biochemical tests including oxidase activity were negative as shown on Table 2. This bacterium utilized organic acids such as acetate, malonate, lactate, succinate and tartarate, and

Table 1. *Morphological observation of the isolated bacterium.*

Type of cells	coccobacillus, single and pairs	
Size of cells	1.0 μm	
Sporulation	negative	
Gram reaction	negative	
Capsule formation	negative	
Swimming motility	negative	
Pigmentation	negative	
Growth on nutrient agar plate	Shape	round
	Surface elevation	ambonate
	Optical characteristic	parcelanous
	Consistency	soft

Table 2. *Biochemical properties of the isolated bacterium.*

Catalase activity	+	Starch hydrolysis	−
Oxidase activity	−	Litmus milk reaction	unchange
Gelatinase activity	−	TSI agar test	
Urease activity	−	slant	alkaline
Indol prodution	−	butt	unchange
H_2S prodution	−	Carbohydrate fermentation	
Methyl red test	−	lactose	−
Nitrate reduction	−	sucrose	−
Voges-proskauer test	−	dextrose	−
MacConkey agar	+	Blood agar	+

some amino acids such as asparagine, arginine, aspartate and alanine as a sole souce of carbon. However it did not utilize any sugar and sugar alcohol examined (Table 3). Although this bacterium could not ferment carbohydrate, it formed a thick white colony on a MacConkey agar plate. In addition to these it also grew on a blood agar plate, but did not show hemolysis. For nitrogen source the bacterium could use ammonium or nitrate salts but it did not require any growth factor. Then the data described above were compared and analyzed with those in Bergey's Mannual of Systematic Bacteriology (Krieg *et al.*, 1984). Based on these examinations the isolated bacterium was identified to be *A. calcoaceticus*.

This bacterium was also tested on an automatic machine for bacterial identification, AVANTAGE microbiology Center, Abbott Laboratories, U.S.A. This machine is generally used for rapid identification of medically important Gram-nega-

Table 3. *Utilization of organic compounds as a sole source of carbon and energy by the isolated bacterium.*

Glucose	−	Sorbitol	−	Itaconate	−
Rhamnose	−	Inisitol	−	Citrate	−
Xylose	−	Acetate	+	Asparate	+
Arabinose	−	Malonate	+	Arginine	+
Lactose	−	Latate	+	Tryptophane	−
Sucrose	−	Succinate	+	Proline	−
Starch	−	Tartarate	+	Aspartate	+
Manitol	−	Oxalate	−	Alanine	+

Table 4. *Specific enzyme activity of the crude extract. The enzymes are known to be involved malonate assimilation.*

Protein	*Pseudomonas fluorescens*		*Acinetobacter calcoaceticus*		
	glucose*	malonate*	LB-broth	succinate*	malonate*
Malonate transport protein	?	may exist[a]	?	?	?
Malonyl-CoA synthetase	N. D.	15.8[b]	N. D.	0.2	9.9
Malonyl-CoA decarboxylase	N. D.	2.5[c]	–	–	–
Isocitrate lyase	N. D.	121.0[d]	N. D.	23	130
Malate synthase	N. D.	103.4[e]	N. D.	100	190

Specific activity (unit/mg protein)

a, b, c, d, e represent the references sited as follow. a, Lee and Kim, 1983 b, Kim and Bang, 1985 c, Kim *et al.*, 1979 d, Jang and Kim, 1982 e, Chae and Kim, 1984
*: represent the compound as a sole carbon source N. D. : not detectable.

tive bacilli. The test result showed that this bacterium was identified to be *A. calcoaceticus* var Lowffi (99.9%) or *P. maltophiloa* (0.09%). On this machine the test on malonate was negative. But it required longer 4 hours to induce the malonate assililation system. These result, therefore, strongly support that the bacterium is *A. calcoaceticus*. So it was named as *A. calcoaceticus* var. Kim.

Metabolic pathway of malonate assimilation in this bacterium

Although the utilization of malonate as a sole carbon source by many microorganisms has been known, its metabolic pathway has not been clearly elucidated yet. Recently Kim and his collaborators isolated a new enzyme, malonyl-CoA synthetase and proposed that malonate is assimilated through the pathway: malonate → malonyl-CoA → acetyl-CoA → glyoxylate cycle in *P. fluorescens* grown on malonate. So they isolated and studied enzymes involved in the pathway, such as malonyl-CoA synthetase, malonyl-CoA decarboxylase, isocitrate lyase and malate synthase from *P. fluorescens*. And they found that all of the enzymes were inducible. So it was interesting to known whether this metabolic pathway is also applicable in other biological system. Therefore *A. calcoaceticus* var Kim which could rapidly grow on malonate medium was isolated from soil. If malonate is assimilated through the metabolic pathway described above, in *A. calcoaceticus* the enzyme such as malonyl-CoA synthetase, malonyl-CoA decarboxylase, isocitrate lyase and malate synthase, might be induced. In order to test this possiblity *A. calcoaceticus* was grown on succinate or malonate as a sole carbon and the cell extracts was prepared. As shown on Table 4 malonyl-CoA synthetase, isocitrate lyase and malate synthase activity were induced in the cells grown on malonate. These results suggest that malonate may be also assimilated in *A. calcoaceticus* through the metabolic pathway proposed in *P. fluorescens*. However it is not clear yet how the cells grown on succinate keep, even if it was low, the enzyme activities.

적 요

Malonate를 유일한 탄소원으로 활용할 수 있는 세균을 토양으로부터 분리하였다. 이 세균은 형태, 배양, 생리 그리고 생화학적 연구를 통하여 ***Acinetobacter calcoaceticus***임이 확인되었다. 이 미생물을 malonate를 유일한 탄소원으로 하는 배지에서 배양하였을 경우, malonyl CoA synthetase, isocitrate lyase 및 malate synthase가 유도되었다. 따라서 이 미생물에서도 ***Pseudomonas fluorescens***에서 제안되었던 대사경로 즉, malonate→malonyl CoA→acetyl CoA→glyoxalate cycle을 통하여 malonate를 이용하는 것으로 판단된다.

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